

1 **Two independent pathways for self recognition in *Proteus mirabilis* are linked by type VI-**
2 **dependent export**

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1 **Abstract**

2 Swarming colonies of the bacterium *Proteus mirabilis* are capable of self recognition and
3 territorial behavior. Swarms of independent *P. mirabilis* isolates can recognize each other as
4 foreign and establish a visible boundary where they meet; by contrast, genetically identical
5 swarms merge. The *ids* genes, which encode self-identity proteins, are necessary but not
6 sufficient for this territorial behavior. Here we have identified two new gene clusters: one (*idr*)
7 encodes *rhs*-related products and another (*tss*) encodes a putative type VI secretion (T6S)
8 apparatus. The Ids and Idr proteins function independently of each other in extracellular transport
9 and in territorial behaviors; however, these self-recognition systems are linked via this type VI
10 secretion system. The T6S system is required for export of select Ids and Idr proteins. Our results
11 provide a mechanistic and physiological basis for the fundamental behaviors of self recognition
12 and territoriality in a bacterial model system.

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15 **Importance**

16 Our results support a model in which self recognition in *P. mirabilis* is achieved by the
17 combined action of two independent pathways linked by a shared machinery for export of
18 encoded self-recognition elements. These proteins together form a mechanistic network for self
19 recognition that can serve as a foundation for examining the prevalent biological phenomena of
20 territorial behaviors and self recognition in a simple, bacterial model system.

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22

1 **Introduction**

2 The ability to differentiate self from nonself is a behavior observed throughout biology,
3 from animals to single-celled organisms. Self recognition has been hypothesized to be a
4 cornerstone aspect of territorial behavior, i.e., a preference for kin and aggressiveness toward
5 non-kin (1). Multiple implementations of self-recognition capability have been described in a
6 growing set of bacteria, including *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*,
7 *Escherichia coli*, *Paenibacillus dendritiformis*, and *Myxococcus xanthus* (2-9). In both *P.*
8 *aeruginosa* and *V. cholerae*, the type VI secretion (T6S) system mediates the exchange of toxins
9 between neighboring cells; kin selection occurs through the strain-specific expression of an
10 antitoxin to the T6S-mediated toxins, which are usually strain- or species-specific effector
11 molecules (3-5). Similarly, contact-dependent inhibition (CDI) in *E. coli* and *Dickeya dadantii* is
12 achieved through the direct exchange of toxin-encoding peptides that are selectively targeted to
13 inhibit growth of non-isogenic strains; these non-isogenic strains do not express the requisite
14 neutralizing antitoxin (7, 10-12). It has been proposed that these CDI toxins are linked to *rhs*
15 (*rearrangement hotspot*) sequences in bacteria (11). While the molecular mechanisms of these
16 systems are beginning to be described, the native environmental and physiological role for self
17 recognition in bacteria is poorly understood.

18 In the model system *P. mirabilis*, a Gram-negative bacterium and causative agent of
19 urinary tract infections, self recognition is necessary for territorial behavior. Migrating
20 populations, or “swarms,” of independent *P. mirabilis* isolates can recognize each other as
21 foreign and establish a macroscopically visible boundary (of up to three millimeters) where they
22 meet. By contrast, genetically identical swarms merge, forming a single, larger swarm (2). This
23 behavior indicates that *P. mirabilis* populations are capable of distinguishing self from nonself.

1 *P. mirabilis* infections have been described as clonal and as a consequence of infection by the
2 host's endogenous strain (13, 14).

3 We previously reported that *P. mirabilis* populations with mutations in the *ids* operon,
4 consisting of *idsABCDEF*, do not merge with the wild-type parent, indicating a loss of the ability
5 to correctly recognize self (15, 16). More specifically, we found that IdsD and IdsE encode
6 strain-specific self-identity determinants in *P. mirabilis*. Strains in which either *idsD* or *idsE* are
7 absent form a territorial boundary with an otherwise genetically identical parent strain, and this
8 behavior is not rescued by expression of *idsD* and/or *idsE* alleles from a foreign strain (15). This
9 differs from the other four Ids proteins (IdsA, IdsB, IdsC, and IdsF), which we found do not
10 confer strain-specific self-identity as their substitution with alleles from a foreign strain does not
11 alter boundary formation (15). The Ids proteins, however, are necessary but not sufficient for self
12 recognition and subsequent boundary formation in *P. mirabilis*.

13 To fully understand and model self-recognition behavior in bacteria, we need to know the
14 core components and how they interact with one another. Indeed, the full set of proteins involved
15 in self recognition in *P. mirabilis*, as well as their cellular location and the interconnections
16 between them, were previously unknown. Moreover, the role of the Ids proteins, and of self
17 recognition in general, in social behaviors outside of boundary formation has yet to be examined.
18 Here, we have characterized core molecular networks for self recognition in one strain of *P.*
19 *mirabilis*, as well as the interconnections between these proteins.

20

21 **Results**

22 **Self recognition requires two gene clusters, *tss* and *idr*, in addition to the *ids* genes.**

1 We sought to ascertain the full set of genes necessary for self recognition by searching
2 for mutants that display a different territorial boundary formation phenotype from the wild-type
3 strain and/or an *ids*-deficient mutant strain. To this end, we generated a library of roughly 13,000
4 single-insertion transposon mutants in the wild-type strain BB2000, representing an
5 approximately three-fold coverage of its genome. Then we screened each mutant from the library
6 by swarming it against a mutant lacking the *ids* operon (Δ *ids*) and against other mutants from the
7 transposon library, which served as proxies for nonself and self populations, respectively. We
8 isolated mutants that either merged with all strains or formed boundaries with Δ *ids* and each
9 other (Figure 1A). Seven mutants were pursued: five that merged with both Δ *ids* and the wild-
10 type parent (“all-merge”) and two that formed boundaries with both Δ *ids* and the wild-type (“no-
11 merge”) (Figure 1B). We had isolated an additional no-merge mutant in a previous self-
12 recognition screen (15). The isolated mutant strains, like the wild-type parent and Δ *ids*, formed
13 boundaries with the independent *P. mirabilis* wild-type strain HI4320 (Figure 1B). The eight
14 insertion sites represented by these recovered mutants map to two adjacent, divergently oriented
15 gene clusters.

16 The insertions in the all-merge mutants map to a single 17-gene cluster, *tssA-Q*, located
17 from base pairs 938,609 to 916,585 (Figure 1C, NCBI accession number BankIt1590180
18 BB2000 CP004022). The sequence of *tssA-Q* reveals similarities to genes encoding core
19 components of the *Vibrio cholerae* type VI secretion (T6S) system, including the membrane
20 proteins *icmF*, *dotU*, and *sciN*, as well as the ATPase *clpV* (17). This is the sole locus containing
21 these T6S proteins in the BB2000 genome. To confirm the phenotype associated with *tssA-Q*, we
22 introduced *tssN* (*icmF*) and the three downstream genes, *tssOPQ*, into a low-copy plasmid where
23 gene expression is controlled by the region directly upstream of *tssA*; we transformed this

1 plasmid, pLW100, into a *tssN*-deficient mutant (*tssN*^{*}) in which *tssN* is disrupted by a
2 transposon insertion. The four *tss* genes, *tssNOPQ*, were included on pLW100, because the *tssN*
3 mutation likely disrupts expression of the downstream genes. The plasmid pLW100
4 complements the *tssN* mutation; the resultant strain forms a boundary with Δ *ids* (Figure 1B). We
5 did not see complementation with a plasmid containing solely *tssN* using the same promoter
6 region, suggesting that a disruption in *tssO*, *tssP*, or *tssQ* may also be responsible for the all-
7 merge phenotype and that the upstream promoter is not contributing to the complementation
8 phenotype (see Supporting Information). Therefore, we conclude that disruption of T6S function
9 is responsible for the all-merge phenotype.

10 The no-merge mutants contain transposon insertions in three separate genes of a
11 previously uncharacterized five-gene locus, located from base pairs 940,506 to 949,474, that we
12 name *idr* for *i*dentit*y* recognition (Figure 1D). The first gene, *idrA*, shares high sequence
13 similarity with *idsA* (98%) and the T6S-related gene *hcp*, whereas the second gene, *idrB*, has
14 some sequence similarity to *idsB* (50%) and the T6S-related gene *vgrG* (Figure 1D). The *idrB*-
15 deficient mutant strain (*idrB*^{*}) in which *idrB* is disrupted by a transposon insertion serves as the
16 *idr*-deficient strain throughout our studies. The remaining genes, *idrC*, *idrD*, and *idrE*, are
17 predicted to encode polypeptides of unknown function. The *idrD* gene contains *rhs* sequences.
18 Some genes containing *rhs* sequences have been shown to encode antibacterial toxins (11).

19 We observe that the *ids*, *idr*, and *tss* gene clusters are all present in the genome of the
20 independent strain HI4320 (18). The Ids proteins share greater than 97% sequence identity
21 among strains, except for IdsD and IdsE, which share 96% and 93% sequence identity,
22 respectively (15). The polypeptides encoded by the *tss* locus are highly similar (over 97%
23 sequence identity) between strains BB2000 and HI4320 (Figure 1C). However, the *idr* locus

1 differs in both nucleotide sequence and gene content between strains BB2000 and HI4320,
2 suggesting that the *idr* locus encodes as-yet uncharacterized strain-specific factors necessary for
3 self recognition (Figure 1D).

4

5 **The *ids*, *tss*, and *idr* loci are each critical for competition on surfaces.**

6 We next examined the role of each gene cluster in self recognition and territorial
7 behaviors. We predicted that self-recognition capability likely provides an increased ability to
8 survive against other organisms. As self recognition-dependent boundary formation in *P.*
9 *mirabilis* is principally apparent on surfaces, we investigated whether loss of self-recognition
10 capability decreases a population's ability to compete on surfaces. In equal initial ratios, we
11 mixed cells of the parent BB2000, which is fully capable of self recognition, with those of either
12 the Δ *ids*, *tssN**, or *idrB** mutant strains, all of which are deficient in one or more self-recognition
13 protein. We placed each mixed population on a nutrient surface in a single spot from which the
14 population migrated outward as a single swarm. Then we analyzed for dominance by measuring
15 whether the mixed population merged with either a pure swarm of parent BB2000 or of an
16 isolated swarm of the tested mutant strain. The parent BB2000 prevailed in virtually every mixed
17 population (Figure 2A).

18 To determine how the parent strain achieves dominance, we sampled for the presence of
19 the parent and mutant strains at discrete locations within the swarm of the mixed population.
20 Notably, parent BB2000 cells migrated to the periphery of the swarm more rapidly than any of
21 the mutant strains (Figure 2B). None of the mutant strains have a motility defect, as compared to
22 BB2000, when migrating alone (Figure 1). Therefore, loss of self-recognition capability
23 diminishes a population's relative rate of movement to, and dominance of, the leading edge of a

1 swarm colony when growing with an otherwise genetically identical strain fully capable of self
2 recognition (Figure 2B).

3 We next assessed how the BB2000 parent and mutant strains fared in competition with
4 the independent wild-type *P. mirabilis* strain HI4320. In similar assays for dominance as
5 described above, we mixed an equal ratio of HI4320 and BB2000 cells and then placed the
6 mixed population onto a nutrient surface in a spot from which the cells migrated outwards as a
7 single swarm. We measured for dominance by examining whether the swarm of the mixed
8 population formed a boundary with an adjacent pure swarm of either HI4320 or of BB2000.
9 Most mixtures of HI4320 and BB2000 yielded boundaries with the neighboring HI4320 swarm
10 but would merge with the BB2000 swarm, indicating that BB2000 cells dominated at the leading
11 edges of mixed populations (Figure 2C). Likewise, mixtures of HI4320 and the *Δids* mutant
12 strain primarily formed boundaries with a pure HI4320 swarm but merged with a pure *Δids*
13 swarm, indicating that the *Δids* strain was dominant in these mixed population and that the *ids*
14 genes are not needed for competition between strains (Figure 2C). By contrast, mixtures of
15 HI4320 with either the *tssN** or *idrB** mutant strain primarily yielded swarms that merged with a
16 pure HI4320 swarm but formed boundaries with pure swarms of the *tssN** or *idrB** mutant
17 strain, respectively, indicating that HI4320 dominated in these mixed populations (Figure 2C).
18 The presence of the Idr and T6S proteins, but not the Ids proteins, is therefore advantageous in
19 competitions against the independent strain HI4320. Further, the Idr and Ids proteins have
20 discrete roles in competitions; while Ids and Idr proteins are necessary for competitions with the
21 parent strain, only Idr proteins are involved in competition with foreign strains.

22

23 **Type VI secretion is required for export of components involved in self recognition.**

1 The phenotypes observed during the competition assays suggest a dynamic connection
2 between these three gene clusters that together contribute to self recognition and territorial
3 behaviors. Since T6S is needed for the export of proteins in other bacterial systems, we predicted
4 that self-recognition products in *P. mirabilis* are likely exported from the cell via this system. As
5 such, we examined the secretion profiles of the wild-type, Δids , *idrB**, and *tssN** strains for
6 proteins involved in self recognition using liquid chromatography-tandem mass spectrometry
7 (LC-MS/MS). We detected the self-identity determinant protein IdsD, as well as IdsA and IdsB,
8 in the extracellular fraction of the wild-type *P. mirabilis* strain BB2000 but not in that of the Δids
9 mutant strain (Figure 3A). None of the remaining Ids proteins were present in any of the
10 extracellular fractions by LC-MS/MS analysis. The newly identified IdrA and IdrB proteins were
11 also present in the extracellular fractions for both the wild-type and the Δids mutant strains,
12 indicating that export of the Idr proteins is independent of the Ids proteins (Figure 3A).
13 Conversely, IdsA, IdsB, and IdsD, as well as IdrA, were detected by LC-MS/MS analysis in
14 supernatant isolated from the *idrB** mutant strain, providing further support that the Ids and Idr
15 proteins likely function independently in export from the cell (Figure 3A).

16 We readily observed IdsA and IdrA in the extracellular fraction of the wild-type strain as
17 discrete bands in a Coomassie Blue-stained protein gel. We excised these bands and confirmed
18 by LC-MS/MS that they were indeed IdsA and IdrA (Figure 3B). Only a single polypeptide band
19 corresponding to the molecular weight of IdrA was present in the Δids extracellular fraction,
20 confirming the LC-MS/MS results (Figure 3B). By contrast, neither IdsA nor IdrA were visible
21 in the extracellular fraction of the *tssN** mutant strain (Figure 3B). Indeed, neither Ids nor Idr
22 proteins were detected above background levels in the supernatant of the *tssN** mutant strain
23 analyzed by LC-MS/MS (see Supporting Information).

1 To further confirm the LC-MS/MS data, we attached a FLAG epitope to the C-terminus
2 of IdsA in a low-copy plasmid containing the entire *ids* operon with its native promoter, resulting
3 in plasmid pLW101, and then introduced this plasmid construct into the Δ *ids* strain, into wild-
4 type BB2000, and into the *tssN** mutant strain. In these plasmid-carrying strains, IdsA-FLAG
5 was absent in the extracellular fraction of the *tssN** mutant strain, but was present in that of the
6 Δ *ids* and wild-type strains, as detected by western blot (Figure 3C). Of note, IdsA-FLAG was
7 detected not only in the supernatant, but also on the surface of intact BB2000 carrying pLW101
8 cells (see Supporting Information). The lack of extracellular IdsA-FLAG in the *tssN** mutant
9 strain was not due to reduced production, as IdsA-FLAG was present at equivalent levels in the
10 whole cell extracts of all plasmid-carrying cells (Figure 3C).

11 We performed a similar western blot analysis using custom-raised antibodies to IdsB.
12 IdsB was detected at equivalent levels in the whole cell extracts of the wild-type, *idrB**, and
13 *tssN** strains, but was only detected in the supernatant fractions of the wild-type and *idrB**
14 strains (Figure 3D). Unfortunately, we were unable to directly localize epitope-tagged variants of
15 IdsD *in vivo*, perhaps due to low expression of IdsD and/or to steric hindrance of the epitope by a
16 putative identity complex. However, based on the LC-MS/MS and western blot analyses, export
17 of the self-identity determinant protein IdsD, as well as the non-identity determinant proteins
18 IdsA, IdsB, IdrA, and IdrB, requires a functional T6S system. Moreover, export of the Ids
19 components is independent of the Idr components and vice-versa.

20

21 **Discussion**

22 Here we report the discovery of two additional gene clusters that together with the *ids*
23 operon comprise a network of self-recognition genes (Figure 4A). One locus, *idr*, encodes

1 proteins necessary for merger with the parent BB2000 strain, while the other locus, *tss*, is a type
2 VI secretion system that mediates export of Ids and Idr proteins. Significantly, we found that
3 multiple Ids and Idr proteins are exported from the cell, including the self-identity determinant
4 protein, IdsD.

5 The Idr and Ids proteins represent two separate mechanisms for self recognition. Export
6 of the Idr proteins is independent of the *ids* gene cluster, and likewise, export of the Ids proteins
7 is not dependent on the *idr* gene cluster. Moreover, strains with mutations in the *ids* or *idr* genes
8 have different phenotypes in intraspecies competitions. The Ids proteins, which are only needed
9 for competition with the parent strain, encode strain-specific self-identity determinants (15).
10 Interestingly, the *idrD* gene contains *rhs* sequences, which are commonly found in bacterial
11 species. Recent research has implicated that these *rhs*-encoding proteins, as well as proteins
12 involved in contact-dependent inhibition such as CdiA in *E. coli*, encode toxin elements in the C-
13 terminal domain (6, 11, 19). However, the *rhs*-containing proteins may also encode for adhesion
14 molecules because they share some sequence similarity to YD-repeat-containing teneurin
15 proteins (11). Either of these *idrD* proposed functions could explain why the *idr* genes are
16 required for increased competition (and/or population migration) against foreign strains.

17 Indeed, we demonstrate that the self-recognition capability of *P. mirabilis* provides a
18 competitive advantage for the population specifically on surfaces. We did not observe similar
19 advantages when wild type was grown with self-recognition mutant strains in liquid (see
20 Supporting Information). Growth on surfaces induces a broad developmental change in *P.*
21 *mirabilis* where increased cell-cell contact yields increased population-wide coordination that is
22 integral to migration and outward expansion of the swarm (reviewed in (20, 21)). Perhaps the

1 behavior of self recognition is most beneficial in environments where social interactions are
2 more frequent and, thus potentially, more impactful.

3 Our research supports a model in which *P. mirabilis* self recognition involves the display
4 of self-identity proteins that are likely interpreted via a direct physical interaction with other
5 cells; this communication then yields a self versus nonself assessment that guides whether
6 boundaries are formed between populations (Figure 4B). Some self-identity proteins are likely
7 displayed on or near the cell surface, as physical contact between cells is required for boundary
8 formation (22). This extracellular exposure may serve to communicate a cell's identity
9 represented by the self-identity determinant molecules, IdsD and IdsE, during interactions with
10 neighboring cells (15, 16). Indeed, Ids and Idr proteins are transported out of the cell via the T6S
11 system and are either transported into the neighboring cell or localize on the cell surface (see
12 Supporting Information). However, we have not yet found evidence for the transfer of self-
13 recognition proteins into a neighboring cell.

14 We propose that boundary formation can result from the actions of a single population,
15 which has queried on a cell-cell level, whether the neighboring cell is self or not. For each
16 population of *P. mirabilis* strain BB2000, “self” is defined by the combined actions of the Ids
17 and Idr pathways. Self recognition occurs when both the expected cognate Ids and Idr proteins
18 are present in (or on) the neighboring cell, ultimately resulting in merger with the neighboring
19 swarm (Figure 4B). By contrast, we predict that boundary formation results from the absence of
20 the cognate Ids and Idr self-determinants in the neighboring cell (Figure 4B). Both the Ids and
21 Idr proteins likely initiate downstream signaling pathways that are altered depending on whether
22 the interactions are with cognate or non-cognate Ids and Idr proteins, respectively.

23 This two-part network for self recognition appears analogous to aspects of the innate

1 immune system and indeed has many parallels to the immune surveillance of natural killer (NK)
2 cells. In current models for NK cell activity, the presence of self cells (i.e., of one's own
3 organism) is conveyed by the combined detection of two surface receptors (an activation-
4 receptor ligand and Class I MHC), resulting in no killing of the self cell. By contrast, the absence
5 of either receptor leads to the NK cell's determination of an absence of self and the subsequent
6 killing of the non-self (or receptor-deficient) cell, as reviewed in (23). Intriguingly, these results
7 in *P. mirabilis* further support the idea that cellular self recognition is a behavior shared amongst
8 many levels of biological complexity.

9 While the capability for self recognition is broadly found, it remains unclear why and
10 how bacteria utilize this ability. In *P. mirabilis*, self recognition is necessary for territorial
11 expansion when interacting with competing non-isogenic populations. Recently, other research
12 groups have shown that type VI secretion systems confer a fitness advantage in interbacterial and
13 interkingdom competitions, likely through transport of small molecules, but their role in
14 intraspecies interactions is only beginning to emerge (3-5, 24-31). Our demonstration that a T6S
15 system functions directly in self recognition-dependent territoriality expands the set of known
16 applications for this widely conserved export machinery. We still need to explore the
17 mechanisms of T6S in *P. mirabilis* and its relative functional capabilities as compared to T6S
18 systems described in other bacteria. Importantly, we still need to understand the dynamics of Idr
19 and Ids protein-protein interactions within and between cells. Indeed, the Ids, Idr, and T6S
20 proteins together form a mechanistic foundation for examining the basic biological phenomena
21 of territoriality and self recognition in a bacterial model system.

22

23

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6

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9

1 **Materials and Methods**

2 **Bacterial strains and media.** All strains are listed in Table 1. *Escherichia coli* strains were
3 maintained on LB agar and *Proteus mirabilis* strains were maintained on LSW- agar (32). *P.*
4 *mirabilis* was grown on CM55 Blood Agar Base agar (Remel Inc., Lenexa, KS) for swarm
5 colony growth. For broth cultures, all strains were grown in LB broth under aerobic conditions at
6 37°C. Antibiotics were used at the following concentrations: 15 µg/ml tetracycline (Tet); 100
7 µg/ml rifampicin (Rif); 50 µg/ml kanamycin (Kn); 35 µg/ml chloramphenicol (Cm) for *E. coli*
8 and 50 µg/ml for *P. mirabilis*. All media contained antibiotics appropriate for selection or
9 maintenance of plasmids.

10

11 **Plasmid construction.** The *tssN* (*icmF*) complementation plasmid pLW100 encodes the last four
12 genes of the *tss* gene cluster (*tssN* through *tssQ*) under the transcriptional control of the proposed
13 promoter contained in the region immediately upstream of the *tss* gene cluster. This plasmid was
14 constructed in two steps: the 1200 basepairs upstream of *tssA* was amplified by Polymerase
15 Chain Reaction (PCR) from the BB2000 genome and inserted into the pBBR1-*NheI* (15) plasmid
16 using the Infusion HD system (Clontech Laboratories, Mountain View, CA); genes *tssN*, *tssO*,
17 *tssP*, and *tssQ* were then PCR-amplified and inserted into the above plasmid (with Infusion HD),
18 resulting in pLW100. To construct pLW101, which is the plasmid encoding IdsA-FLAG, a
19 FLAG epitope (N-DYKDDDDK-C) was inserted immediately before the *idsA* stop codon in the
20 *pids_{BB}* plasmid (15) using Quikchange site-directed mutagenesis (Agilent Technologies, Santa
21 Clara, CA). Plasmids were propagated in *E. coli* XL10-Gold and then conjugated into *P.*
22 *mirabilis* via mating with *E. coli* S17-1λpir carrying the target plasmid as described earlier (15).

23

1 **Swarm boundary assay.** Cells were inoculated from overnight or mid-logarithmic cultures with
2 an inoculation needle onto the surface of CM55 agar. Swarm plates were incubated for 18 - 24
3 hours at 37°C and screened by eye for the presence or absence of a visible boundary.

4
5 **Transposon library construction and screen.** A library of *P. mirabilis* transposon insertion
6 mutants was generated by mating *P. mirabilis* strain BB2000 with *E. coli* strain SM10λpir
7 carrying pUTmini-Tn5-Cm as described previously (15). Matings were performed on LSW⁻ agar
8 plates in the absence of selection for 8 - 16 hours, spread on 22 cm x 22 cm LSW⁻ Tet+Cm agar
9 trays (Genetics/Molecular Devices, UK), and incubated at 37°C for 24 - 36 hours. Colonies were
10 picked using a robotic colony picker (Qbot/Molecular Devices, Genetix, UK) and arrayed into
11 96-well master plates. In total, 12,960 transposon insertion mutants were arrayed from 96-well
12 master plates onto swarm agar trays (Nunc Omnitray, Nalge Nunc International, Rochester, NY)
13 using the gridding head of the robotic colony picker. The swarm agar trays were arrayed in one
14 of two patterns: 1) the mutants alone were arrayed and screened for boundary formation between
15 independent mutants, or 2) the mutants were arrayed alternating with the *Δids* mutant. After 24
16 and 48 hours, each mutant was scored for boundary formation or merger with neighboring
17 colonies. From the initial high-throughput, robotic screen of ~26,000 interactions, 192 potential
18 mutants were selected for further re-testing. Potential mutants were then examined in individual
19 swarm boundary assays against the BB2000 parent, the *Δids* mutant strain, and wild-type *P.*
20 *mirabilis* strain HI4320 (33) to confirm the phenotype. Of those tested, 21 mutant strains were
21 confirmed. Eight mutants contained disruptions in eight different loci, six strains contained
22 mutations in the *ids* locus, and the remaining mutants contained disruptions in the *tss* (five) and
23 *idr* (two) loci.

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Mapping the transposon insertion sites. Arbitrary PCR was used to map the sites of the mini-Tn5-Cm transposon insertions as described previously (34-36). Briefly, genomic DNA was isolated from each transposon mutant of interest by phenol chloroform extraction, and the transposon insertion sites were amplified using Vent Polymerase (New England Biolabs, Ipswich, MA) and primers Tn5Ext and ARB6 for the first round, then oNS054 (5'-TTCACACAGGAAACAGCTATGACCGCATTAATACTAGCGAGG -3') and ARB2 for the second round. Samples were treated with ExoSAP-IT (New England Biolabs, Ipswich, MA) between rounds and prior to sequencing. Sanger sequencing was performed using primer oNS056 (5'-TTCACACAGGAAACAGCTATGACC -3') via Genewiz, Inc. (South Plainfield, NJ). Results were mapped against the HI4320 (18) and the BB2000 (NCBI accession number BankIt1590180 BB2000 CP004022) genome sequences using ViroBLAST (37).

Sequence alignments. The predicted polypeptide sequences for the *ids*, *tss*, and *idr* gene clusters were compared between independent *P. mirabilis* strains BB2000 and HI4320. Percent identities for the entire peptide were calculated in pairwise comparisons using ClustalW2 (38, 39).

Surface competitions. To observe the spatial distribution of co-swarming *P. mirabilis* strains over time, BB2000 c. pBBR2-GFP (16) was competed against BB2000, Δ *ids*, *tssN**, or *idrB**. Overnight cultures were normalized to an OD₆₀₀ of 0.1. Competing strains were mixed together in a 1:1 ratio, and 0.5 μ L of each co-culture was spotted onto the center of a CM55 agar plate. After incubating first at room temperature for 22 hours and then at 37°C for 6 hours, each swarm consisted of four swarm rings and was patched using a half-plate 48-prong device onto selective

1 plates that could detect the marked BB2000 strain (LSW⁻ Kn) and, when applicable, the mutant
2 strain (LSW⁻ Cm). Select swarms (i.e. BB2000 versus BB2000) were also plated non-selectively
3 onto LSW⁻ agar. To determine which strain was dominant in surface competitions, overnight
4 cultures, normalized to OD₆₀₀ of 1.0, of BB2000 were mixed at a 1:1 ratio with those of *Δids*,
5 *tssN**, or *idrB**. Mixed populations were inoculated onto CM55 agar using an inoculation
6 needle; monocultures of the boundary indicator strains were inoculated approximately 1 cm
7 away. After overnight incubation at 37°C, the presence or absence of boundaries between swarm
8 was used to assess strain dominance at the leading edge. Dominance of BB2000 was assessed as
9 the merger of mixed populations with a mono-swarm of BB2000; dominance of the mutant was
10 assessed as a merger with a mono-swarm of itself, or in the case of the *tssN** strain, the *Δids*
11 strain.

12 For competition between independently derived strains, the surface competition described
13 above was repeated using the *P. mirabilis* wild-type strain HI4320 competed against BB2000,
14 *Δids*, *tssN**, or *idrB**, with the modifications that a mono-culture of HI4320 was used as the
15 indicator strain on each plate instead of BB2000 and dominance of HI4320 was assessed as the
16 merger of mixed populations with the HI4320 mono-swarm. For all assays, unclear boundaries
17 were marked as neither.

18
19 **TCA precipitation.** Overnight cultures were diluted to an OD₆₀₀ of 0.1 in fresh LB+Kn and
20 grown at 37°C with shaking to an OD₆₀₀ of 3.5 - 4.5. For whole cell extracts, 1 ml of culture was
21 centrifuged and the pellet was resuspended in 100 μl SDS-PAGE sample buffer. For supernatant
22 samples, 30 ml of culture was clarified by centrifugation and filter sterilized (0.22 μm filters).
23 The filtered supernatant was treated with trichloroacetic acid (10% final concentration) and

1 incubated on ice for 30 minutes. Precipitated proteins were collected by centrifugation, washed
2 twice with pre-chilled 100% acetone, dried, and resuspended in 100 μ L 2X SDS-PAGE sample
3 buffer.

4
5 **Protein sequence analysis by LC-MS/MS.** TCA-precipitated samples were analyzed by
6 electrophoresis using 10% SDS-PAGE gels and then stained with Coomassie Blue. Gel regions
7 of interest were excised and analyzed by liquid chromatography-mass spectrometry/mass
8 spectrometry (LC-MS/MS) by the Taplin Biological Mass Spectrometry Facility (Harvard
9 Medical School, Boston, MA). The unique peptide results for the Ids and Idr proteins are in
10 Tables S1, S2, S3, and S4.

11
12 **Antiserum preparation.** Polyclonal antiserum against residues Cys713-Ala723 of IdsB was
13 raised in rabbits according to the standard protocols (Covance Research Products, Denver, CO).

14
15 **Gel electrophoresis and western blot.** Protein samples were separated by gel electrophoresis
16 using 15% tris-tricine gels and were either stained with Coomassie Blue or transferred to
17 nitrocellulose for western blot analysis. Western blot membranes were probed with primary
18 antibody (either 1:5000 mouse anti-FLAG, Sigma-Aldrich, Allentown, PA; 1:1000 mouse anti-
19 Sigma70, Thermo Scientific, West Palm Beach, FL; or, 1:1000 rabbit anti-IdsB antiserum) for 1
20 hour, with secondary antibody (1:5000 goat anti-mouse-HRP, KPL, Gaithersburg, MD; 1:5000
21 goat anti-rabbit-HRP, KPL, Gaithersburg, MD) for 1 hour, and visualized using Immun-Star
22 HRP Luminol/Enhancer (Bio-Rad, Hercules, CA) and the ChemiDoc XRS (Bio-Rad, Hercules,
23 CA).

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1 **Figure Legends**

2 **Figure 1. The *tss* and *idr* genes are necessary for self recognition.** (A) Diagrammatic
3 representations of the boundary behavior patterns exhibited by mutants isolated in the screen,
4 matched with the swarm plates below. (B) Swarm agar plates inoculated with *P. mirabilis* strains
5 exhibited the boundary formation behavior of two representative mutants isolated from the self-
6 recognition screen: the *tssN** mutant strain that merged with all other BB2000-derived strains
7 (left), the complemented *tssN** mutant strain carrying plasmid pLW100 that formed a boundary
8 with the *Δids* strain (center), and the *idrB** mutant that formed boundaries with all other strains
9 (right). (C) Diagram of the putative type VI secretion (*tss*) gene locus with sites of the transposon
10 insertions, as depicted by lollipops. (D) Diagram of the *idr* gene locus with sites of the
11 transposon insertions, as depicted by lollipops. For (C) and (D), the dark grey shading indicates
12 97% or higher percent identity for the predicted polypeptide sequences of the *tss* and *idr* genes
13 between strains BB2000 and HI4320; otherwise, specific identities are provided underneath. The
14 dashed box indicates the region of *idrD* that shares sequence similarity between strains BB2000
15 and HI4320. Slanted lines indicate a break in the genomic regions, corresponding to
16 approximately 8 kilobase pairs (kb).

17

18 **Figure 2. Competitions between *P. mirabilis* strains.** (A) Competitions between mutant strains
19 and the parent strain BB2000 on surfaces were initiated at a 1:1 ratio, and the mixed populations
20 were permitted to swarm against either BB2000 or the mutant strain, N = 12. Population
21 dominance was measured as the ability of the mixed swarm to merge with either BB2000,
22 indicating BB2000 dominance, or the mutant strain, indicating dominance of the mutant strain.
23 Unclear boundaries were classified as neither. (B) To observe the spatial distribution of co-

1 swarming *P. mirabilis* strains over time, BB2000 c. pKG101 (16) was competed against
2 BB2000, Δids , *tssN**, or *idrB**. Overnight cultures were normalized to an OD₆₀₀ of 0.1.
3 Competing strains were mixed together in a 1:1 ratio, and 0.5 μ L of each co-culture was spotted
4 onto the center of a CM55 agar plate, N=3. After incubating first at room temperature for 22
5 hours and then at 37°C for 6 hours, each swarm consisted of four swarm rings and was patched
6 using a half-plate 48-prong device onto selective plates that could detect the marked BB2000
7 strain (LSW⁻ Kn) and, when applicable, the mutant strain (LSW⁻ Cm). Swarms of BB2000 versus
8 BB2000 were also plated non-selectively onto LSW⁻ agar. Representative photographs of the
9 swarm plates, after sampling for migration distance, are depicted below. (C) Competitions
10 between BB2000, the BB2000 mutant strains, and an independent strain HI4320 were initiated at
11 a 1:1 ratio, and the mixed populations were swarmed against either a BB2000 mutant strain or
12 HI4320. The BB2000 mutant strain was defined as dominant when there was a merger between
13 the mixed population and the BB2000 mutant strain, while HI4320 was dominant if the mixed
14 population merged with the HI4320 swarm. N = 6 for *tssN**; 12 for Δids and *idrB**; and 18 for
15 BB2000.

16

17 **Figure 3. Proteins involved in self recognition are exported outside of the cell.** (A) LC-
18 MS/MS peptide hits for proteins in the culture supernatants of wild-type BB2000 and the *Δids*
19 mutant strains. *For BB2000 and the *idrB** strains, an additional 6 unique (74 total) and 4 unique
20 (28 total) peptides, respectively, could be assigned to either IdsA or IdrA, due to high similarity
21 of the two proteins. (B) The secretion profiles of the wild-type, *Δids*, and *tssN** strains were
22 examined by gel electrophoresis followed by Coomassie Blue staining. The identity of bands
23 corresponding to IdsA and IdrA were confirmed by LC-MS/MS. (C) Western blots of

1 extracellular secretions (left) and whole cell extracts (right) isolated from strains expressing
2 IdsA-FLAG. The Δids c. $pids_{BB}$ strain was included as a negative control for the FLAG epitope.
3 For Δids expressing IdsA-FLAG *in trans*, the FLAG epitope was engineered in-frame into an
4 expression plasmid that contains the entire *ids* operon under native control. (D) Western blots of
5 extracellular secretions (left) and whole cell extracts (right) isolated from the indicated strains
6 using a polyclonal anti-IdsB antibody. The asterisks mark the size of the expected band.

7
8 **Figure 4. Model for Ids and Idr functional roles in self recognition.** (A) Depicted is a
9 functional flowchart for the roles of the Ids, Idr, and T6S proteins in self recognition and
10 territorial behaviors. A subset of Ids and Idr proteins are primarily exported via a shared T6S
11 system (*tss*) and are necessary for competition on surfaces with the parent strain. Idr proteins are
12 also needed for competition against foreign strains. (B) Our proposed model for self recognition
13 predicts that the combined actions of interactions between cognate Ids and Idr proteins between
14 two neighboring cells result in the determination that self is present, ultimately resulting in the
15 merger of two swarms. Expression of the self-recognition components within the cells is
16 sufficient, though in wild-type strains, some of these components are exported out of the cell by
17 a T6S system. By contrast, absence of one or more of the Ids and Idr self-recognition systems
18 leads to the determination that self is absent and ultimately to boundary formation.

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1 **Table 1. Bacterial strains and plasmids.**

Strain	Genotype	Source
<i>Proteus mirabilis</i>		
BB2000	wild type	(32)
HI4320	wild type	(18, 40)
Δids	$\Delta ids::Cm(R)$	(15)
Δids c. $pids_{BB}$	$\Delta ids::Cm(R)$ carrying a plasmid expressing the <i>ids</i> operon under control of the <i>ids</i> upstream region	(15)
<i>idrB</i> *	<i>idrB::Tn-Cm(R)</i>	This study
<i>idrC</i> *	<i>idrC::Tn-Cm(R)</i>	This study
<i>idrD</i> *	<i>idrD::Tn-Cm(R)</i>	(15)
<i>tssA</i> *	<i>tssA::Tn-Cm(R)</i>	This study
<i>tssB</i> *	<i>tssB::Tn-Cm(R)</i>	This study
<i>tssG</i> *	<i>tssG::Tn-Cm(R)</i>	This study
<i>tssM</i> *	<i>tssM::Tn-Cm(R)</i>	This study
<i>tssN</i> *	<i>tssN::Tn-Cm(R)</i>	This study
BB2000 c. pKG101	wild type carrying a plasmid with Kn(R) and promoter-less <i>gfp</i>	(16)
<i>tssN</i> * c. pLW100	<i>tssN::Tn-Cm(R)</i> carrying a plasmid expressing <i>tssNOPQ</i> under control of the <i>tssA</i> upstream region	This study
BB2000 c. pLW101	wild type carrying a plasmid expressing	This study

	IdsA-FLAG in which a FLAG was engineered to the C-terminus of IdsA in the pids _{BB} vector	
<i>Δids</i> c. pLW101	<i>Δids</i> ::Cm(R) carrying a plasmid expressing IdsA-FLAG in which a FLAG was engineered to the C-terminus of IdsA in the pids _{BB} vector	This study
<i>tssN*</i> c. pLW101	<i>tssN*</i> ::Cm(R) carrying a plasmid expressing IdsA-FLAG in which a FLAG was engineered to the C-terminus of IdsA in the pids _{BB} vector	This study
<i>Escherichia coli</i>		
SM10λpir c. pUTmini-Tn5-Cm	Cm(R)	(41)
S17-1λpir		(41)
XL10-Gold Ultracompetent Cells		Agilent Technologies, Santa Clara, CA

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1 **Supporting Information legends**

2 **Figure S1. Boundary assays with *tssN**-derived strain.** (A) Diagrammatic representations of
3 the boundary behavior patterns exhibited by the indicated strains, matched with the swarm plate
4 to the right. (B) On this swarm agar plate, the *tssN** mutant strain carrying plasmid pLW103,
5 which encodes for *tssN* expression alone, merges with the both *Δids* strain (top) and the parent
6 BB2000 (left), as did the *tssN** mutant strain.

7 Method: The *tssN*-expressing plasmid, pLW103, encodes the *tssN* gene under the transcriptional
8 control of the proposed promoter contained in the region immediately upstream of the *tss* gene
9 cluster. This plasmid was constructed as follows: pLMW100 was digested at NheI and XmaI
10 sites to obtain the vector backbone; gene *tssN* and the 1200 basepairs upstream region were then
11 PCR-amplified from pLMW100 using primers 5'-
12 ATAGCTAGCTCGAGGCCTCTCATTACAGTAGCAATATTGAGAGAAGATT-3' and 5'-
13 ATACCCGGGCCCCGCGGTTAATAAAGCGTTTCAGGTAAACGGA-3'; this product was
14 then digested with NheI and XmaI and ligated with the vector backbone. The plasmid pLW103
15 was then transformed into *E. coli* S17λpir using standard protocols and subsequently conjugated
16 into the *tssN** mutant strain.

17

18 **Figure S2. No clear disadvantage for loss of self-recognition in liquid competitions.** No
19 significant difference was seen between the growth of BB2000 and each self-recognition mutant
20 when grown together in liquid broth after three hours (as measured by a two-tailed t-test, p =
21 0.38, 0.39, and 0.14 for *Δids*, *tssN**, and *idrB**, respectively) and after 20 hours (as measured by
22 a two-tailed t-test, p = 0.22, 0.89, and 0.31 for *Δids*, *tssN**, and *idrB**, respectively). These

1 results suggest that the self-recognition components do not confer a competitive advantage under
2 liquid-grown conditions.

3 Method: The constitutive *lacZ* expression plasmid pLW102 encodes the *lacZ* gene under the
4 transcriptional control of the *fla* and *lac* promoters. This plasmid was constructed as follows:
5 pKG105 (Gibbs et al., 2011) was digested at SacI and AgeI sites to obtain the vector backbone;
6 gene *lacZ* was then PCR-amplified from pQF50 (1) using primers 5'-
7 CATGAGCTCATGAAAGGGAATTCCTGGCC-3' and 5'-
8 TAAACCGGTTTATTTTTGACACCAGACCAACTG-3'; after digesting with SacI and AgeI,
9 this product was then ligated with the vector backbone. The ligation reaction was transformed
10 into Stellar Competent Cells (Clontech Laboratories, Mountain View, CA). The plasmid
11 pLW102 was then transformed into *E. coli* S17 λ pir using standard protocols and subsequently
12 transformed into wild-type BB2000. BB2000 c. pLW102 was competed against BB2000 c.
13 pKG101, *Δids* c. pKG101, *tssN** c. pKG101, or *idrB** c. pKG101. Overnight cultures were back-
14 diluted to OD₆₀₀ of 0.1 in 3 ml LB+Kn and rotated at 37°C until late-log growth. Cultures were
15 then normalized to OD₆₀₀ of 3.5. The competing strains were mixed together in a 1:10
16 BB2000:mutant ratio, back-diluted to a 1:3 in LB+Kn for a total volume of 1.5 ml, and incubated
17 at 37°C while shaking at 225 rpm. After three hours and 20 hours of growth, cells were spotted
18 on non-selective (LSW⁻+Kn and 300 μg/ml X-gal) and, when applicable, selective (LSW⁻+Cm)
19 plates to measure for colony forming units (CFUs). The resultant ratio in CFUs of each strain
20 was compared to the initial inoculation ratio to calculate fold change. Statistical analysis was
21 performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

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Figure S3. Dot blot analysis of IdsA-FLAG. IdsA-FLAG, expressed *in trans* in *P. mirabilis* cells, was found in the supernatant and on the cell surface of wild-type BB2000 and in severely attenuated amounts for the *tssN** mutant strain. The blot on the left was probed with an anti-FLAG antibody primary while an anti-sigma-70 antibody was used to probe the blot on the right as a control for cell lysis. These results confirm that IdsA requires *tssN* for proper export out of the cell as indicated by the LC-MS/MS and western blot analyses. This result suggests that IdsA is normally located and exposed on the cell surface.

Method: Samples for whole cell immunoblots were prepared as described previously (2). Briefly, cell cultures were grown to late-logarithmic phase. “Cell culture” samples were prepared by spotting directly onto nitrocellulose membrane in 5 x 5 µl aliquots for a total of 25 µl in each spot. For “cell surface” samples, the loosely-adhered portion of a cell pellet from 5 ml of cell culture was gently resuspended in 1 ml LB and spotted on the membrane as above. For “lysed cells” samples cells from 10 ml of culture were collected by centrifugation and resuspended in 1 ml lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% triton X-100, 1 mM EDTA, and protease inhibitor cocktail, Roche, Indianapolis, IN). Cells were lysed by vortexing with cell disruptor beads (Electron Microscopy Sciences, Hatfield, PA) and centrifuged to remove cell debris. The soluble fraction was spotted onto the membrane as above. The dot immunoblot was then developed as the above western blots using one of two primary antibodies: mouse anti-FLAG (Sigma-Aldrich, Allentown, PA) or mouse anti-sigma70 (Pierce Biotechnology, Rockford, IL.)

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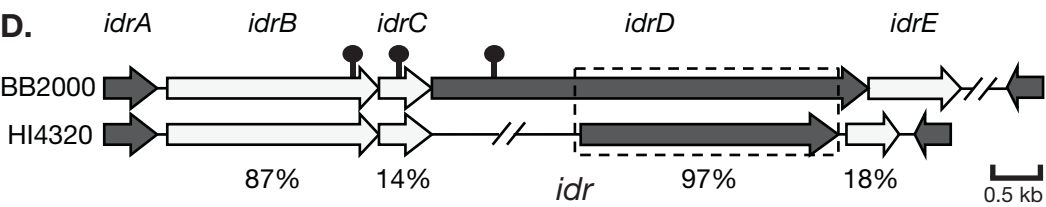
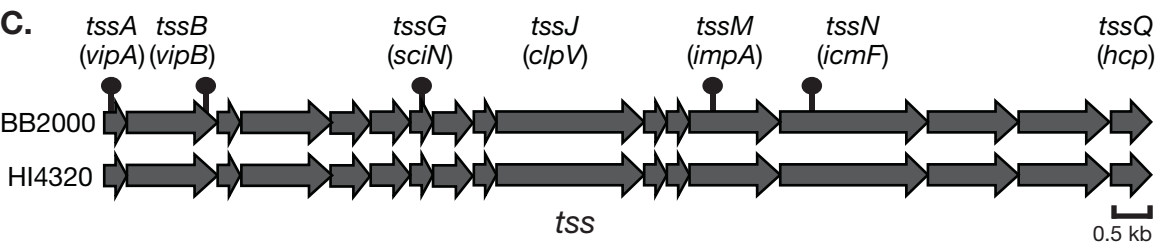
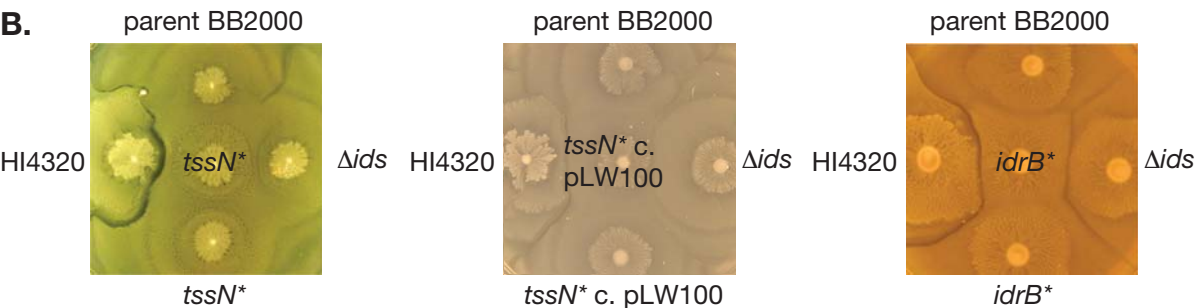
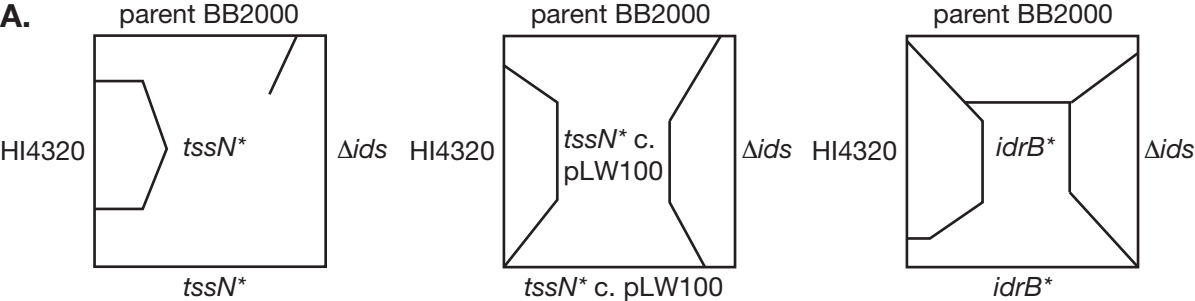
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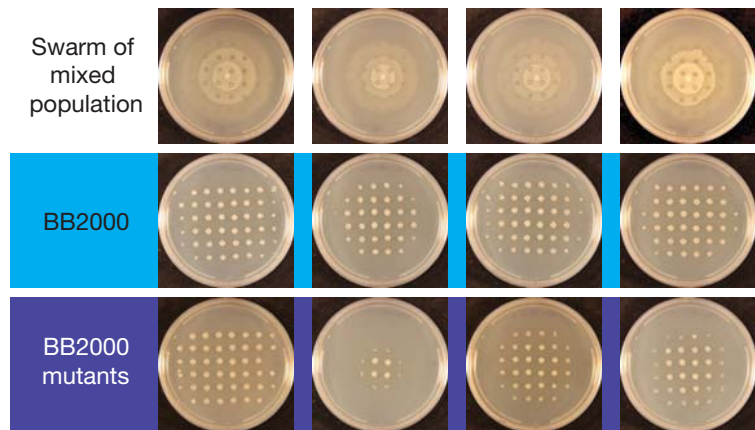
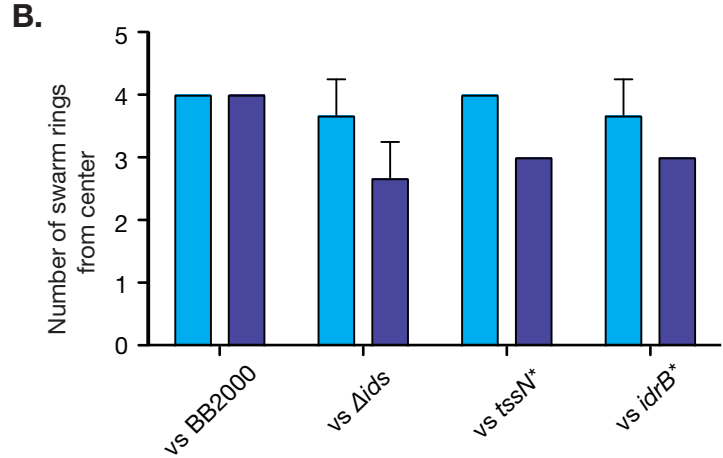
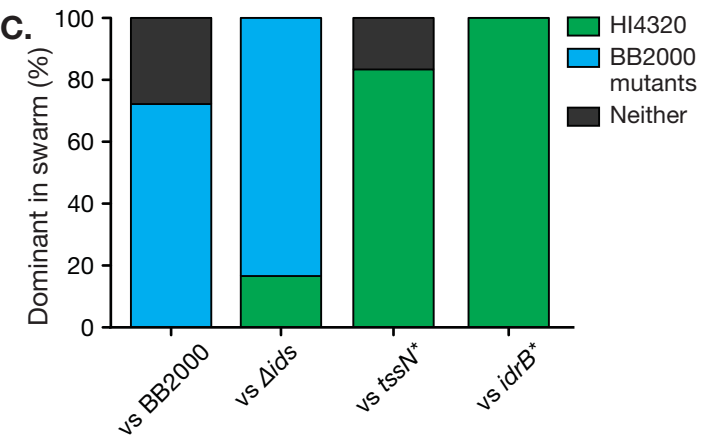
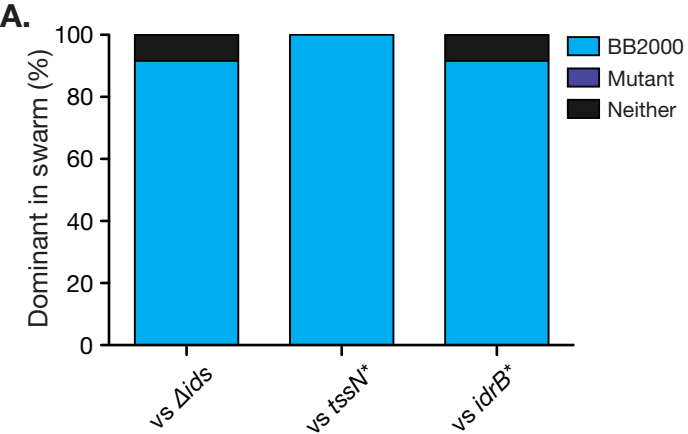
Table S1. For the wild-type parent strain BB2000, listed are the unique peptide results for Ids and Idr proteins, acquired by LC-MS/MS. Peptide fragments that could correspond to either IdsA or IdrA are marked as such. The minimum detection cut-off recommended by the Taplin Biological Mass Spectrometry Facility was three unique peptides.

Table S2. For the *Δids* mutant strain, listed are the unique peptide results for Ids and Idr proteins, acquired by LC-MS/MS. The minimum detection cut-off recommended by the Taplin Biological Mass Spectrometry Facility was three unique peptides.

Table S3 For the *idrB** mutant strain, listed are the peptide results for Ids and Idr proteins, acquired by LC-MS/MS. Peptide fragments that could correspond to either IdsA or IdrA are marked as such. The minimum detection cut-off recommended by the Taplin Biological Mass Spectrometry Facility was three unique peptides.

Table S4. For the *tssN** mutant strain, listed are the unique peptide results for Ids and Idr proteins, acquired by LC-MS/MS. Peptide fragments that could correspond to either IdsA or IdrA are marked as such. Consistent with the dot blots, the *tssN** mutant strain is greatly attenuated for IdsA or IdrA export, though not completely deficient. The *tssN** mutant strain does not export any of the remaining Ids and Idr proteins. The minimum detection cut-off recommended by the Taplin Biological Mass Spectrometry Facility was three unique peptides, which IdrA/IdsA did not achieve for the *tssN** sample.





A. Wild-type BB2000:

Protein	Predicted size (kDa)	No. of unique peptides	No. of total peptides	Percent coverage
IdsA ⁺	18.9	3	18	13.4
IdsB	79.5	8	10	16.3
IdsD	113.7	6	6	5.7
IdrA ⁺	18.9	6	31	39.5
IdrB	80.3	22	32	33.3

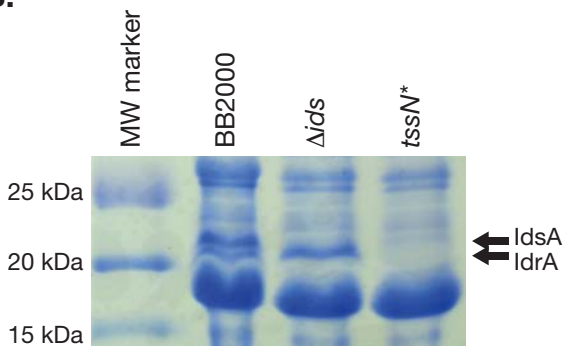
The Δ *ids* mutant strain:

Protein	Predicted size (kDa)	No. of unique peptides	No. of total peptides	Percent coverage
IdrA	18.9	8	134	27.3
IdrB	80.3	14	23	24.4

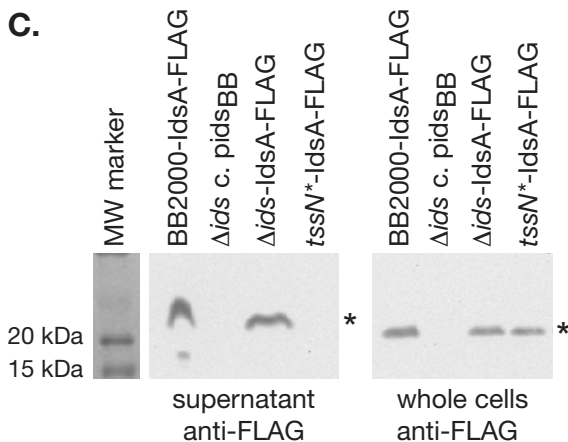
The *idrB*^{*} mutant strain:

Protein	Predicted size (kDa)	No. of unique peptides	No. of total peptides	Percent coverage
IdsA ⁺	18.9	3	13	13.4
IdsB	79.5	9	11	18.4
IdsD	113.7	13	15	13.1
IdrA ⁺	18.9	4	24	35.5

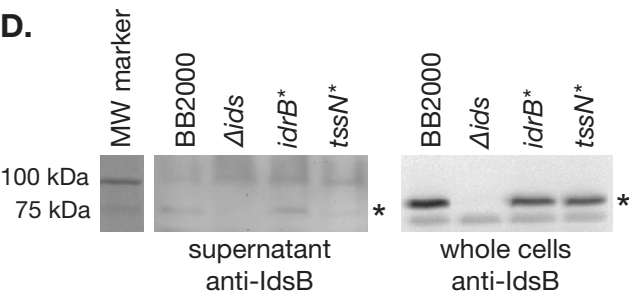
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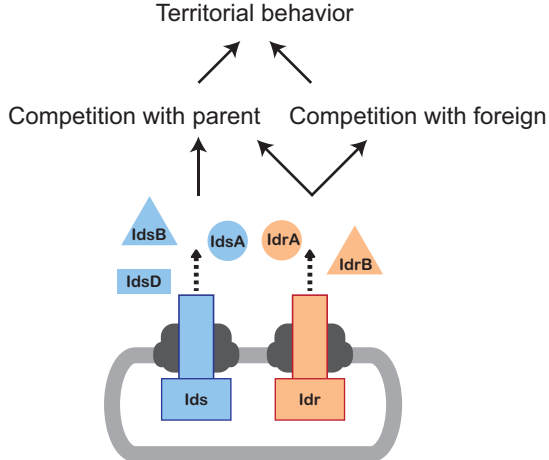
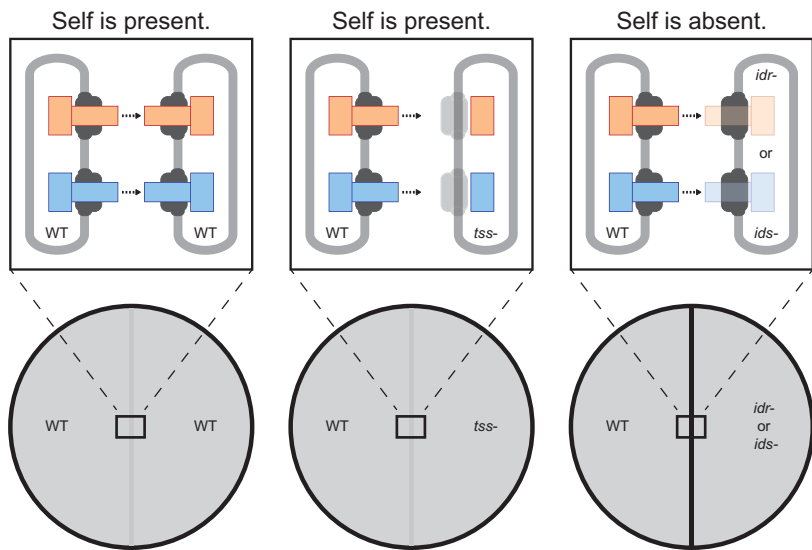


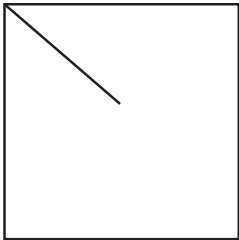
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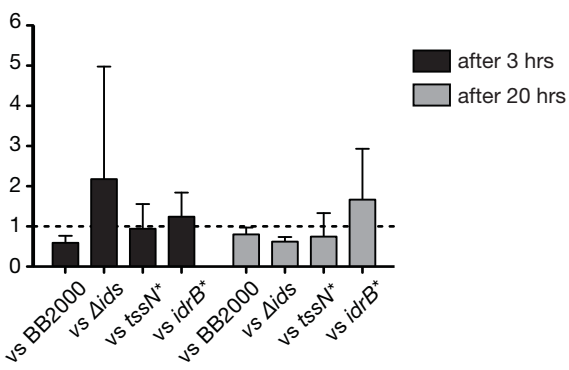
D.



A**B**

A. Δids parent
BB2000*tssN** c.
pLW103*tssN** c. pLW103**B.** Δids parent
BB2000*tssN** c.
pLW103*tssN** c. pLW103

Fold change (final:initial)

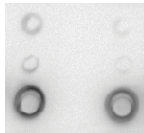


cell culture
cell surface
lysed cells

BB2000-IdsA-FLAG

Δ ids-pids^{BB}

tssN*-IdsA-FLAG

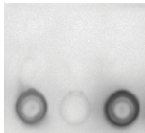


α -FLAG

BB2000-IdsA-FLAG

Δ ids-pids^{BB}

tssN*-IdsA-FLAG



α -sigma70

Table S1.

<u>Reference</u>	<u>XCorr</u>	<u>dCn</u>	<u>dCn2</u>	<u>Ions</u>	<u>Peptide</u>
IdsA	5.3896	0.5549	0.5549	25/32	K.VDWEHTVAGTSGADDWR.A
IdsA	4.2485	0.4309	0.4309	29/68	R.KVDWEHTVAGTSGADDWR.A
IdsA	5.9656	0.4986	0.4986	38/88	R.KVDWEHTVAGTSGADDWRAPLEA.-
IdsB	4.759	0.5298	0.5298	21/30	K.AGSIQLDAQGVTTITGK.I
IdsB	3.7675	0.3475	0.3475	24/60	K.TQYVGHDDSHTVANNR.K
IdsB	2.8448	0.3676	0.3676	15/18	R.DNNIHINHNK.T
IdsB	5.1584	0.4859	0.4859	22/26	R.FEEDAQQQPFNQIR.Y
IdsB	2.3677	0.1035	0.1035	13/20	R.IFTLSNHPSAR.M
IdsB	2.9674	0.2714	0.2714	17/30	R.QVGSATTNC#IELAPGR.I
IdsB	5.0171	0.4794	0.4794	31/68	R.TVQGILAAAEQGNTDGVK.T
IdsB	2.4417	0.3319	0.3319	14/32	R.VAQGWNGDGFQFM*AIPR.V
IdsD	2.0964	0.1035	0.1035	11/18	K.EATILFSESK.L
IdsD	3.1107	0.293	0.293	14/24	K.ESINQNALDNEWK.N
IdsD	1.9879	0.1803	0.1803	10/14	K.ISLTEFVK.L
IdsD	3.6108	0.3934	0.3934	16/20	K.SYQEQNVDATK.G
IdsD	2.4055	0.29	0.29	10/12	K.TFIFFEK.H
IdsD	2.1136	0.3466	0.3466	13/18	K.TPSSAYVLNK.R
IdrA	4.9102	0.3358	0.3358	29/64	K.IDWEHTVAGTSGADDWR.A
IdrA	5.4061	0.4659	0.4659	38/84	K.IDWEHTVAGTSGADDWRAPLEA.-
IdrA	5.6797	0.467	0.467	32/68	R.KIDWEHTVAGTSGADDWR.A

IdrA	4.0785	0.372	0.372	24/88	R.KIDWEHTVAGTSGADDWRAPLEA.-
IdrA or IdsA	4.2545	0.4478	0.4478	20/26	K.ADFTQLIEVSLSYR.K
IdrA or IdsA	2.8423	0.3304	0.3304	14/32	K.AVPLLYNALASGEM*LPK.V
IdrA or IdsA	4.4527	0.431	0.431	34/64	K.AVPLLYNALASGEMLPK.V
IdrA or IdsA	3.0643	0.3506	0.3506	30/80	K.AVPLLYNALASGEM*LPKVELK.W
IdrA or IdsA	3.0339	0.1195	0.1195	19/40	K.AVPLLYNALASGEMLPKVELK.W
IdrA or IdsA	2.2485	0.2758	0.2758	11/12	K.VELKWYR.T
IdrA or IdsA	2.0092	0.1537	0.1537	9/12	R.FTVALNK.A
IdrA or IdsA	5.2513	0.4468	0.4468	31/92	R.FTVALNKAVPLLYNALASGEM*LPK. V
IdrB	3.6976	0.3279	0.3279	16/18	K.DM*NTVVQNDK.G
IdrB	3.3175	0.285	0.285	15/18	K.DMNTVVQNDK.G
IdrB	4.282	0.4147	0.4147	28/60	K.DNNFVRPSYPLSHENK.I
IdrB	2.7468	0.1269	0.1269	13/14	K.GEGFNELR.F
IdrB	4.2695	0.343	0.343	31/64	K.GTTVGANHTETIM*QNQK.I
IdrB	4.9009	0.4954	0.4954	24/28	K.IEQGGQHSHVFESYGR.F

IdrB	4.2312	0.441	0.441	31/60	K.ISVHGTQTTAVQADQK.N
IdrB	1.6257	0.1322	0.1322	9/16	K.QEVFLHAQK.D
IdrB	2.8236	0.2991	0.2991	14/16	K.TLLDEAHVK.A
IdrB	3.2206	0.1876	0.1876	18/32	K.VNGILAGAVQGNTDGVK.T
IdrB	2.2093	0.1234	0.1234	10/12	R.DGVLIRK.V
IdrB	2.6654	0.3208	0.3208	13/16	R.ESAFDFWC#R.L
IdrB	3.6954	0.3472	0.3472	23/60	R.FEDAGGKQEVFLHAQK.D
IdrB	3.1383	0.3473	0.3473	17/26	R.FQLDDEGRPLTQVR.F
IdrB	2.4368	0.3	0.3	14/20	R.HLGLASLTVK.R
IdrB	3.5169	0.2206	0.2206	17/22	R.IFQHQSVPTILK.T
IdrB	6.084	0.4449	0.4449	4	R.IGTGELLDLNM*DGAGPGNLEM*KP DTSTIAQAK.D
IdrB	3.9134	0.1133	0.1133	29/68	R.KVNGILAGAVQGNTDGVK.T
IdrB	1.6251	0.1763	0.1763	8/10	R.LYTTQK.R
IdrB	2.3779	0.129	0.129	14/20	R.NAPPIKFPENK.T
IdrB	3.4027	0.3438	0.3438	23/32	R.VAM*GWSGNGYGFSAPR.I
IdrB	3.2794	0.2957	0.2957	23/32	R.VAMGWSGNGYGFSAPR.I

Table S2.

<u>Reference</u>	<u>XCorr</u>	<u>dCn</u>	<u>dCn2</u>	<u>Ions</u>	Peptide
IdrA	3.7201	0.3646	0.3646	28/64	K.AVPLLYNALASGEM*LPK.V
IdrA	4.4093	0.3502	0.3502	34/64	K.AVPLLYNALASGEMLPK.V
IdrA	4.6002	0.4061	0.4061	26/64	K.IDWEHTVAGTSGADDWR.A
IdrA	4.664	0.4101	0.4101	34/84	K.IDWEHTVAGTSGADDWRAPLEA.-
IdrA	1.769	0.0592	0.0592	8/12	K.VELKWYR.T
IdrA	1.9592	0.1684	0.1684	8/12	R.FTVALNK.A
IdrA	5.6781	0.6324	0.6324	27/34	R.KIDWEHTVAGTSGADDWR.A
IdrA	3.1953	0.2248	0.2248	25/88	R.KIDWEHTVAGTSGADDWRAPLEA.-
IdrB	3.3388	0.3399	0.3399	15/18	K.DM*NTVVQNDK.G
IdrB	4.5883	0.437	0.437	27/60	K.DNNFVRPSYPLSHENK.I
IdrB	2.7321	0.1448	0.1448	13/14	K.GEGFNELR.F
IdrB	4.307	0.3083	0.3083	29/64	K.GTTVGANHTETIM*QNQK.I
IdrB	4.2783	0.3057	0.3057	28/56	K.IEQGGQHSVFESYGR.F
IdrB	3.1619	0.3241	0.3241	27/60	K.ISVHGTQTTAVQADQK.N
IdrB	2.5799	0.1261	0.1261	11/12	K.RDGVLIR.K
IdrB	2.3248	0.2782	0.2782	15/16	R.ESAFDFWC#R.L
IdrB	3.0519	0.352	0.352	16/26	R.FQLDDEGRPLTQVR.F
IdrB	5.8585	0.4843	0.4843	4	R.IGTGELLDLNM*DGAGPGNLEM*KP DTSTIAQAK.D
IdrB	1.6372	0.1415	0.1415	8/10	R.LYTTQK.R

IdrB	2.3593	0.2877	0.2877	13/20	R.NAPPIKFPENK.T
IdrB	4.9851	0.5957	0.5957	24/32	R.VAM*GWSGNGYGFSAPR.I
IdrB	3.4545	0.2977	0.2977	23/32	R.VAMGWSGNGYGFSAPR.I

Table S3.

<u>Reference</u>	<u>XCorr</u>	<u>dCn</u>	<u>dCn2</u>	<u>Ions</u>	<u>Peptide</u>
IdsA	4.9592	0.4513	0.4513	29/64	K.VDWEHTVAGTSGADDWR.A
IdsA	5.7991	0.5746	0.5746	26/34	R.KVDWEHTVAGTSGADDWR.A
IdsA	6.4703	0.5907	0.5907	37/88	R.KVDWEHTVAGTSGADDWRAPLEA.-
IdsB	4.6550	0.4943	0.4943	21/30	K.AGSIQLDAQGVTTITGK.I
IdsB	4.4363	0.3836	0.3836	28/60	K.TQYVGHDDSHTVANNR.K
IdsB	4.8031	0.3950	0.3950	28/92	R.AGISLTYNPQSDTDITDSTATTWR.Y
IdsB	3.5356	0.4250	0.4250	15/18	R.DNNIHINHKN.T
IdsB	5.6329	0.5124	0.5124	22/26	R.FEEDAQQQPFNQIR.Y
IdsB	3.4731	0.4005	0.4005	16/20	R.IFTLSNHPSAR.M
IdsB	2.3558	0.2076	0.2076	9/12	R.SPIDLPK.H
IdsB	6.4621	0.5810	0.5810	25/34	R.TVQGILAAAEQGNTDGVK.T
IdsB	3.6928	0.3932	0.3932	24/32	R.VAQQWNGDGFQFM*AIPR.V
IdsD	2.2796	0.1292	0.1292	12/14	K.DALQVSTK.L
IdsD	3.4800	0.2688	0.2688	16/18	K.DLLEISEQLK.M
IdsD	1.7805	0.0837	0.0837	11/12	K.DPVGYQK.D
IdsD	3.1138	0.3244	0.3244	16/18	K.EATILFSESK.L
IdsD	1.7527	0.0873	0.0873	15/22	K.IAGAVGAALAAR.D
IdsD	2.0824	0.1028	0.1028	12/14	K.IEIIDITK.N
IdsD	4.4499	0.3154	0.3154	22/26	K.IINLGEETAVLIPK.I
IdsD	2.3336	0.2436	0.2436	13/14	K.ISLTEFVK.L

IdsD	3.5650	0.2871	0.2871	17/20	K.LSQTVSSTTLK.F
IdsD	3.6800	0.4398	0.4398	16/20	K.SYQEQNVDATK.G
IdsD	3.4074	0.4669	0.4669	20/32	K.TLEASIPPSINQLLNAK.D
IdsD	2.3196	0.3378	0.3378	15/18	K.TPSSAYVLNK.R
IdsD	2.6735	0.2396	0.2396	14/16	K.VISLIANSK.I
IdrA	5.5520	0.4721	0.4721	24/32	K.IDWEHTVAGTSGADDWR.A
IdrA	5.2084	0.5535	0.5535	37/84	K.IDWEHTVAGTSGADDWRAPLEA.-
IdrA	3.5020	0.1120	0.1120	29/68	R.KIDWEHTVAGTSGADDWR.A
IdrA	4.2771	0.4223	0.4223	31/88	R.KIDWEHTVAGTSGADDWRAPLEA.-
IdrA or IdsA	4.0383	0.4488	0.4488	17/26	K.ADFTQLIEVLSYR.K
IdrA or IdsA	4.4752	0.5319	0.5319	22/32	K.AVPLLYNALASGEM*LPK.V
IdrA or IdsA	4.7170	0.5144	0.5144	20/32	K.AVPLLYNALASGEMLPK.V
IdrA or IdsA	1.5866	0.0996	0.0996	8/12	R.FTVALNK.A

Table S4.

<u>Reference</u>	<u>XCorr</u>	<u>dCn</u>	<u>dCn2</u>	<u>Ions</u>	<u>Peptide</u>
IdrA or IdsA	5.3089	0.5427	0.5427	24/32	K.AVPLLYNALASGEM*LPK.V
IdrA or IdsA	5.3944	0.5881	0.5881	25/32	K.AVPLLYNALASGEM*LPK.V
IdrA or IdsA	4.2235	0.4096	0.4096	21/32	K.AVPLLYNALASGEM*LPK.V
IdrA or IdsA	5.1316	0.5971	0.5971	22/32	K.AVPLLYNALASGEMLPK.V
IdrA or IdsA	5.479	0.5245	0.5245	24/32	K.AVPLLYNALASGEMLPK.V