Two Independent Pathways for Self-Recognition in *Proteus mirabilis* Are Linked by Type VI-Dependent Export

Larissa M. Wenren, Nora L. Sullivan,* Lia Cardarelli, Alecia N. Septer, Karine A. Gibbs

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, USA

* Present address: Nora L. Sullivan, W. M. Keck Science Department, Claremont McKenna, Pitzer and Scripps Colleges, Claremont, California, USA.

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

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

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Address correspondence to Karine A. Gibbs. kagibbs@mcb.harvard.edu.

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

In the model system *P. mirabilis*, a Gram-negative bacterium and causative agent of urinary tract infections, self-recognition is necessary for territorial behavior. Migrating populations, or swarms, of independent *P. mirabilis* isolates can recognize each other as foreign and establish a macroscopically visible boundary (of up to 3 mm) where they meet. In contrast, genetically identical swarms merge, forming a single, larger swarm (2). This behavior indicates that *P. mirabilis* populations are capable of distinguishing self from nonself. *P. mirabilis* infections have been described as clonal and as a consequence of infection by the host's endogenous strain (13, 14).

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

To fully understand and model self-recognition behavior in bacteria, we need to know the core components and how they interact with one another. Indeed, the full set of proteins involved

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in self-recognition in *P. mirabilis*, as well as their cellular location and the interconnections between them, were previously unknown. Moreover, the role of the Ids proteins, and of self-recognition in general, in social behaviors outside boundary formation has yet to be examined. Here, we have characterized core molecular networks for self-recognition in one strain of *P. mirabilis*, as well as the interconnections between these proteins.

RESULTS

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

The insertions in the all-merge mutants map to a single 17-gene cluster, *tssA-Q*, located from base pair 938,609 to 916,585 (Fig. 1C) (NCBI accession number BankIt1590180 BB2000 CP004022). The sequence of *tssA-Q* reveals similarities to genes encoding core components of the *Vibrio cholerae* type VI secretion (T6S) system, including the membrane protein genes *icmF*, *dotU*, and *sciN*, as well as the ATPase gene *clpV* (17). This is the sole locus containing these T6S proteins in the BB2000 genome. To confirm the phenotype associated with *tssA-Q*, we introduced *tssN* (*icmF*) and the three downstream genes, *tssOPQ*, into a low-copy-number plasmid where gene expression is controlled by the region directly upstream of *tssA*; we transformed this plasmid, pLW100, into a *tssN*-deficient mutant (*tssN**) in which *tssN* is disrupted by a transposon insertion. The genes *tssNOPQ* were included on pLW100, because the *tssN* mutation likely disrupts expression of the downstream genes. The plasmid pLW100 complements the *tssN* mutation; the resultant strain forms a boundary with Δ*ids* (Fig. 1B). We did not see complementation with a plasmid containing solely *tssN* using the same promoter region, suggesting that a disruption in *tssO*, *tssP*, or *tssQ* may also be responsible for the all-merge phenotype and that the upstream promoter is not contributing to the complementation phenotype (see the supplemental material). Therefore, we conclude that disruption of T6S function is responsible for the all-merge phenotype.

The no-merge mutants contain transposon insertions in three separate genes of a previously uncharacterized five-gene locus, located from base pair 940,506 to 949,474, that we name *idr* for "identity recognition" (Fig. 1D). The first gene, *idrA*, shares high sequence similarity with *idsA* (95%) and the T6S-related gene *hcp*, whereas the second gene, *idrB*, has some sequence similarity to *idsB* (55%) and the T6S-related gene *vgrG* (Fig. 1D). The *idrB*-deficient (*idrB**) mutant strain in which *idrB* is disrupted by a transposon insertion served as the *idr*-deficient strain throughout our studies. The remaining genes, *idrC*, *idrD*, and *idrE*, are predicted to encode polypeptides of unknown function. The *idrD* gene contains *rhs* sequences. Some genes containing *rhs* sequences have been shown to encode antibacterial toxins (11).

We observe that the *ids*, *idr*, and *tss* gene clusters are all present in the genome of the independent strain HI4320 (18). The Ids proteins share more than 97% sequence identity among strains, except for IdsD and IdsE, which share 96% and 93% sequence identity, respectively (15). The polypeptides encoded by the *tss* locus are highly similar (over 97% sequence identity) between strains BB2000 and HI4320 (Fig. 1C). However, the *idr* locus differs in both nucleotide sequence and gene content between strains BB2000 and HI4320, suggesting that the *idr* locus encodes as-yet uncharacterized strain-specific factors necessary for self-recognition (Fig. 1D).

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

To determine how the parent strain achieves dominance, we sampled for the presence of the parent and mutant strains at discrete locations within the swarm of the mixed population. Notably, parent BB2000 cells migrated to the periphery of the swarm more rapidly than any of the mutant strains (Fig. 2B). None of the mutant strains have a motility defect, compared to BB2000, when migrating alone (Fig. 1). Therefore, loss of self-recognition capability diminishes a population's relative rate of movement to, and dominance of, the leading edge of a swarm colony when it is growing with an otherwise genetically identical strain fully capable of self-recognition (Fig. 2B).

We next assessed how the BB2000 parent and mutant strains fared in competition with the independent wild-type *P. mirabilis* strain HI4320. In similar assays for dominance as described above, we mixed an equal ratio of HI4320 and BB2000 cells and then placed the mixed population onto a nutrient surface in a spot from which the cells migrated outward as a single swarm. We measured for dominance by examining whether the swarm of the mixed population formed a boundary with an adjacent pure swarm of either HI4320 or BB2000. Most mixtures of HI4320 and BB2000 yielded boundaries with the neighboring HI4320 swarm but merged with the BB2000 swarm, indicating that BB2000 cells

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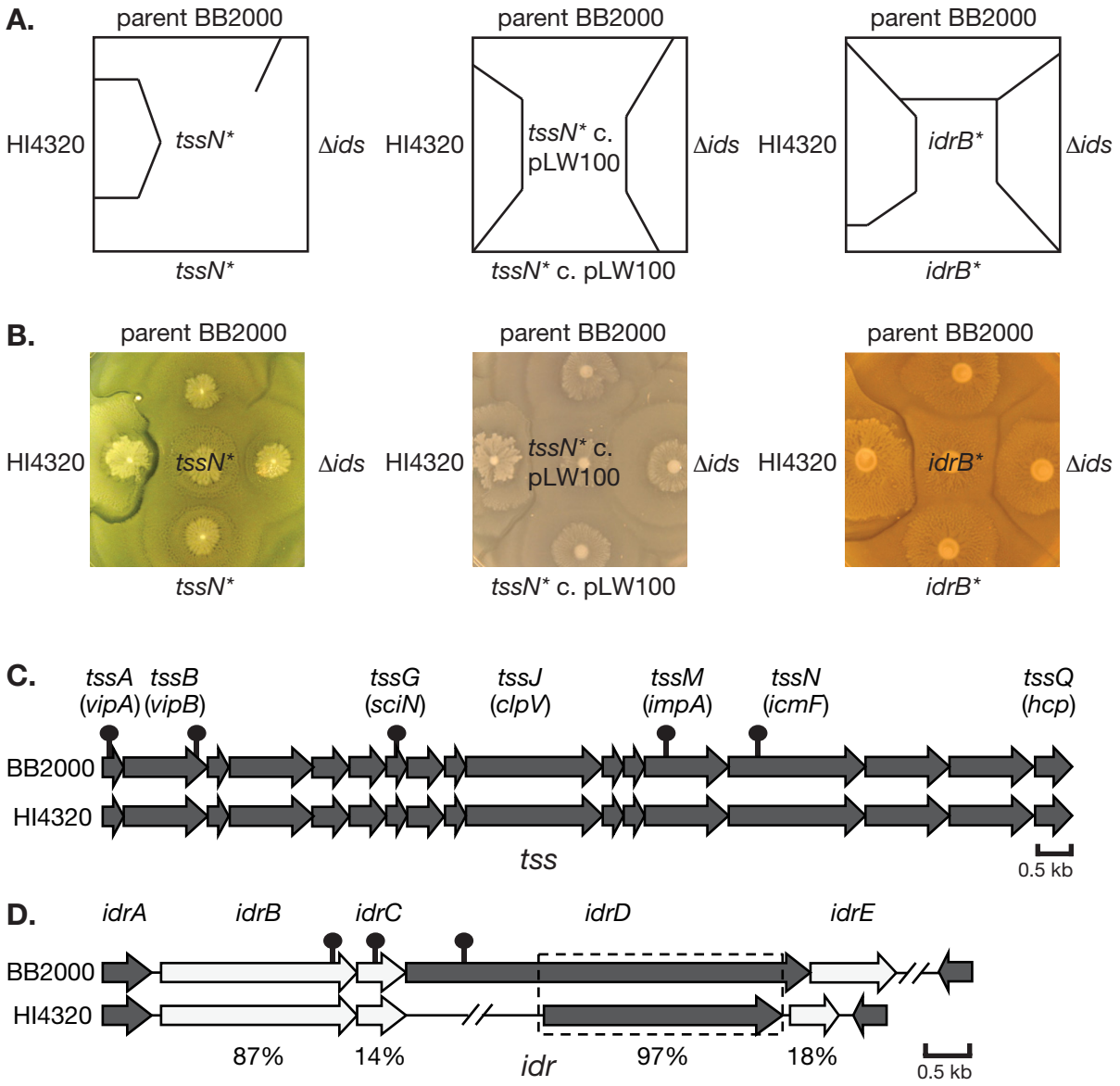

FIG 1 The *tss* and *idr* genes are necessary for self-recognition. (A) Diagrammatic representations of the boundary behavior patterns exhibited by mutants isolated in the screen, matched with the swarm plates below. (B) Swarm agar plates inoculated with *P. mirabilis* strains exhibited the boundary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

dominated at the leading edges of mixed populations (Fig. 2C). Likewise, mixtures of HI4320 and the Δ*ids* mutant strain primarily formed boundaries with a pure HI4320 swarm but merged with a pure Δ*ids* swarm, indicating that the Δ*ids* strain was dominant in these mixed population and that the *ids* genes are not needed for competition between strains (Fig. 2C). In contrast, mixtures of HI4320 with either the *tssN** or *idrB** mutant strain primarily yielded swarms that merged with a pure HI4320 swarm but formed boundaries with pure swarms of the *tssN** or *idrB** mutant strain, respectively, indicating that HI4320 dominated in these mixed populations (Fig. 2C). The presence of the Idr and T6S proteins, but not the Ids proteins, is therefore advantageous in competitions against the independent strain HI4320. Further, the Idr and Ids proteins have discrete roles in competitions; while Ids and Idr proteins are necessary for competitions with the parent strain, only Idr proteins are involved in competition with foreign strains.

Type VI secretion is required for export of components involved in self-recognition. The phenotypes observed during the competition assays suggest a dynamic connection between these

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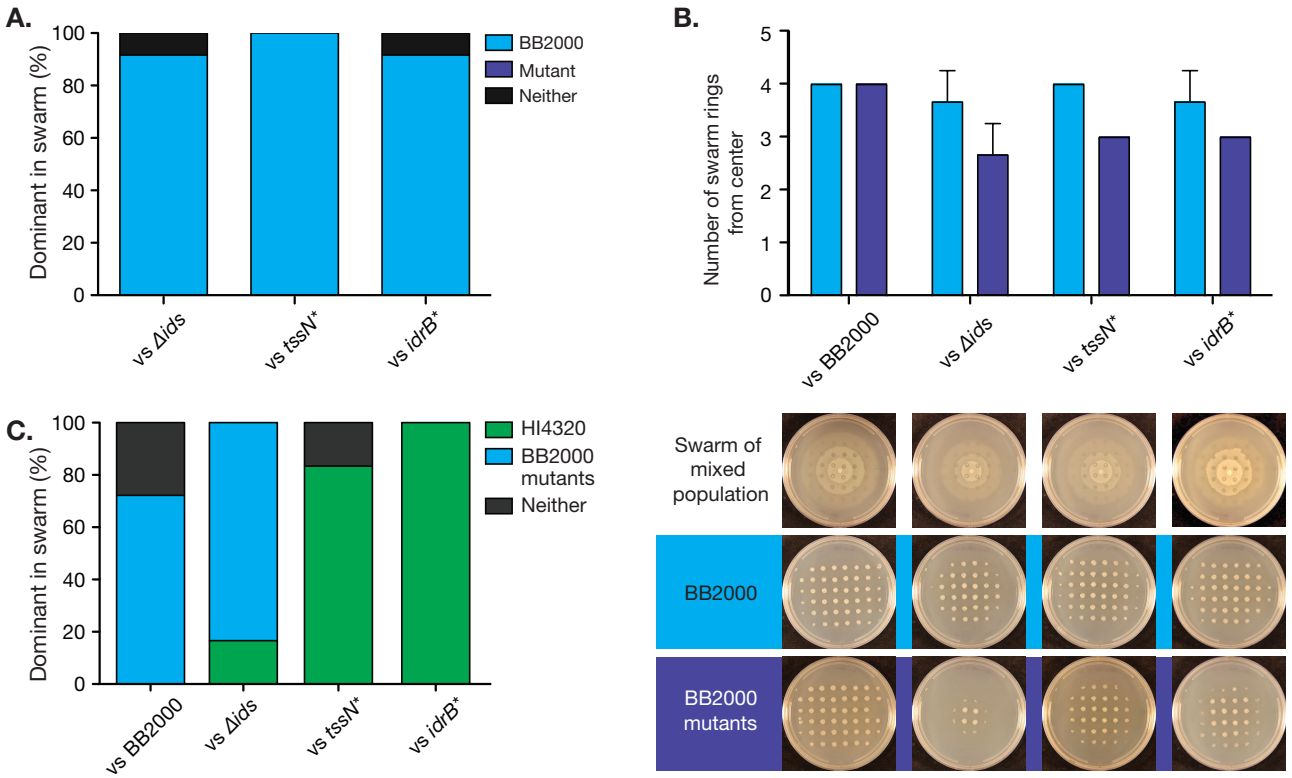

FIG 2 Competitions between *P. mirabilis* strains. (A) Competitions between mutant strains and the parent strain BB2000 on surfaces were initiated at a 1:1 ratio, and the mixed populations were permitted to swarm against either BB2000 or the mutant strain ($n = 12$). Population dominance was measured as the ability of the mixed swarm to merge with either BB2000, indicating BB2000 dominance, or the mutant strain, indicating dominance of the mutant strain. Unclear boundaries were classified as "neither." (B) To observe the spatial distribution of coswarming *P. mirabilis* strains over time, BB2000 c. pKG101 (16) was competed against BB2000 or the Δ*ids*, *tssN**, or *idrB** mutant. Overnight cultures were normalized to an OD_{600} of 0.1. Competing strains were mixed in a 1:1 ratio, and 0.5 μl of each coculture was spotted onto the center of a CM55 agar plate ($n = 3$). After incubation first at room temperature for 22 h and then at 37°C for 6 h, each swarm consisted of four swarm rings and was patched using a half-plate 48-prong device onto selective plates that could detect the marked BB2000 strain (LSW^- Kn) and, when applicable, the mutant strain (LSW^- Cm). Swarms of BB2000 versus BB2000 were also plated nonselectively onto LSW^- agar. Representative photographs of the swarm plates, after sampling for migration distance, are depicted in the lower portion. (C) Competitions between BB2000, the BB2000 mutant strains, and an independent strain, HI4320, were initiated at a 1:1 ratio, and the mixed populations were swarmed against either a BB2000 mutant strain or HI4320. The BB2000 mutant strain was defined as dominant when the mixed population merged with the BB2000 mutant strain, while HI4320 was dominant if the mixed population merged with the HI4320 swarm. $n = 6$ for the *tssN** strain, 12 for the Δ*ids* and *idrB** strains, and 18 for BB2000.

three gene clusters that together contribute to self-recognition and territorial behaviors. Since T6S is needed for the export of proteins in other bacterial systems, we predicted that self-recognition products in *P. mirabilis* are likely exported from the cell via this system. As such, we examined the secretion profiles of the wild-type, Δ*ids*, *idrB**, and *tssN** strains for proteins involved in self-recognition using liquid chromatography-tandem mass spectrometry (LC-MS/MS). We detected the self-identity determinant protein IdsD, as well as IdsA and IdsB, in the extracellular fraction of the wild-type *P. mirabilis* strain BB2000 but not in that of the Δ*ids* mutant strain (Fig. 3A). None of the remaining Ids proteins were present in any of the extracellular fractions by LC-MS/MS analysis. The newly identified IdrA and IdrB proteins were also present in the extracellular fractions for both the wild-type and the Δ*ids* mutant strains, indicating that export of the Idr proteins is independent of the Ids proteins (Fig. 3A). Conversely, IdsA, IdsB, and IdsD, as well as IdrA, were detected by LC-MS/MS analysis in supernatant isolated from the *idrB** mutant strain, providing further support for the idea that the Ids and Idr proteins likely function independently in export from the cell (Fig. 3A).

We readily observed IdsA and IdrA in the extracellular fraction of the wild-type strain as discrete bands in a Coomassie blue-stained protein gel. We excised these bands and confirmed by LC-MS/MS that they were indeed IdsA and IdrA (Fig. 3B). Only a single polypeptide band corresponding to the molecular weight of IdrA was present in the Δ*ids* extracellular fraction, confirming the LC-MS/MS results (Fig. 3B). In contrast, neither IdsA nor IdrA was visible in the extracellular fraction of the *tssN** mutant strain (Fig. 3B). Indeed, neither Ids nor Idr proteins were detected above background levels in the supernatant of the *tssN** mutant strain analyzed by LC-MS/MS (see the supplemental material).

To further confirm the LC-MS/MS data, we attached a FLAG epitope to the C terminus of IdsA in a low-copy-number plasmid containing the entire *ids* operon with its native promoter, resulting in plasmid pLW101, and then introduced this plasmid construct into the Δ*ids* strain, into wild-type BB2000, and into the *tssN** mutant strain. In these plasmid-carrying strains, IdsA-FLAG was absent in the extracellular fraction of the *tssN** mutant strain but was present in that of the Δ*ids* and wild-type strains, as detected by Western blotting (Fig. 3C). Of note, IdsA-FLAG was detected not only in the supernatant but also on the surface of

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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 *Δids* 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

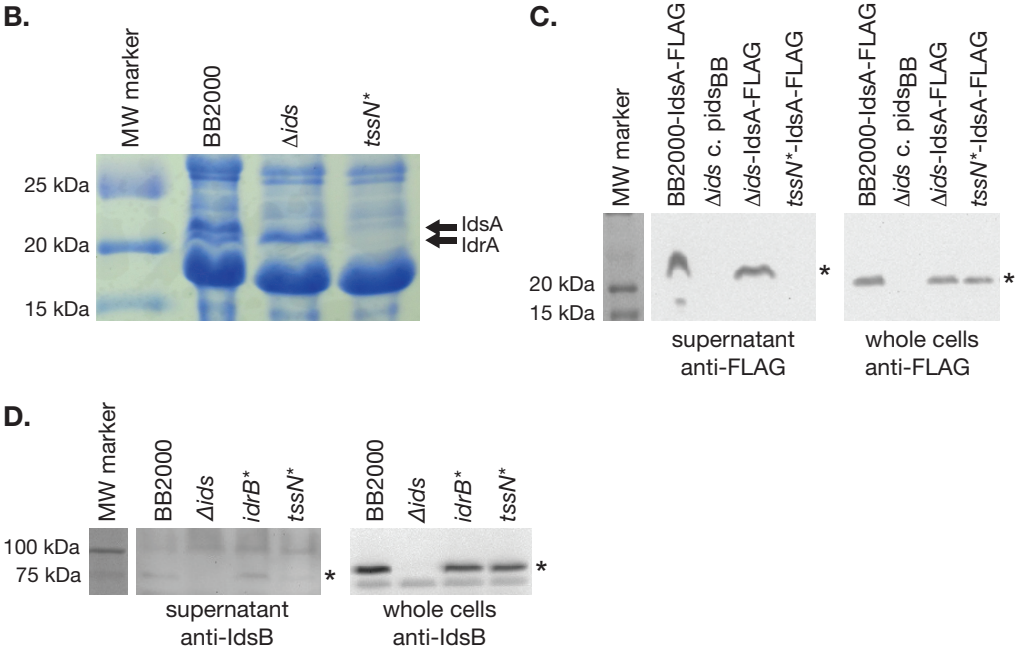

FIG 3 Proteins involved in self-recognition are exported outside the cell. (A) LC-MS/MS peptide hits for proteins in the culture supernatants of wild-type BB2000 and the Δ*ids* mutant strains. For BB2000 and the *idrB** strains, an additional 6 unique (74 total) and 4 unique (28 total) peptides, respectively, could be assigned to either IdsA or IdrA, due to high similarity of the two proteins (indicated by a plus sign). (B) The secretion profiles of the wild-type, Δ*ids*, and *tssN** strains were examined by gel electrophoresis followed by Coomassie blue staining. The identity of bands corresponding to IdsA and IdrA were confirmed by LC-MS/MS. (C) Western blots of extracellular secretions (left) and whole-cell extracts (right) isolated from strains expressing IdsA-FLAG. The Δ*ids* c. $pids_{BB}$ strain was included as a negative control for the FLAG epitope. For Δ*ids* expressing IdsA-FLAG in *trans*, the FLAG epitope was engineered in frame into an expression plasmid that contains the entire *ids* operon under native control. (D) Western blots of extracellular secretions (left) and whole-cell extracts (right) isolated from the indicated strains using a polyclonal anti-IdsB antibody. The asterisks mark the size of the expected band.

intact BB2000 carrying pLW101 cells (see the supplemental material). The lack of extracellular IdsA-FLAG in the *tssN** mutant strain was not due to reduced production, as IdsA-FLAG was present at equivalent levels in the whole-cell extracts of all plasmid-carrying cells (Fig. 3C).

We performed a similar Western blot analysis using custom-raised antibodies to IdsB. IdsB was detected at equivalent levels in the whole-cell extracts of the wild-type, *idrB**, and *tssN** strains but was detected only in the supernatant fractions of the wild-type and *idrB** strains (Fig. 3D). Unfortunately, we were unable to directly localize epitope-tagged variants of IdsD *in vivo*, perhaps due to low expression of IdsD and/or to steric hindrance of the epitope by a putative identity complex. However, based on the LC-MS/MS and Western blot analyses, export of the self-identity determinant protein IdsD, as well as the non-identity determinant proteins IdsA, IdsB, IdrA, and IdrB, requires a functional T6S

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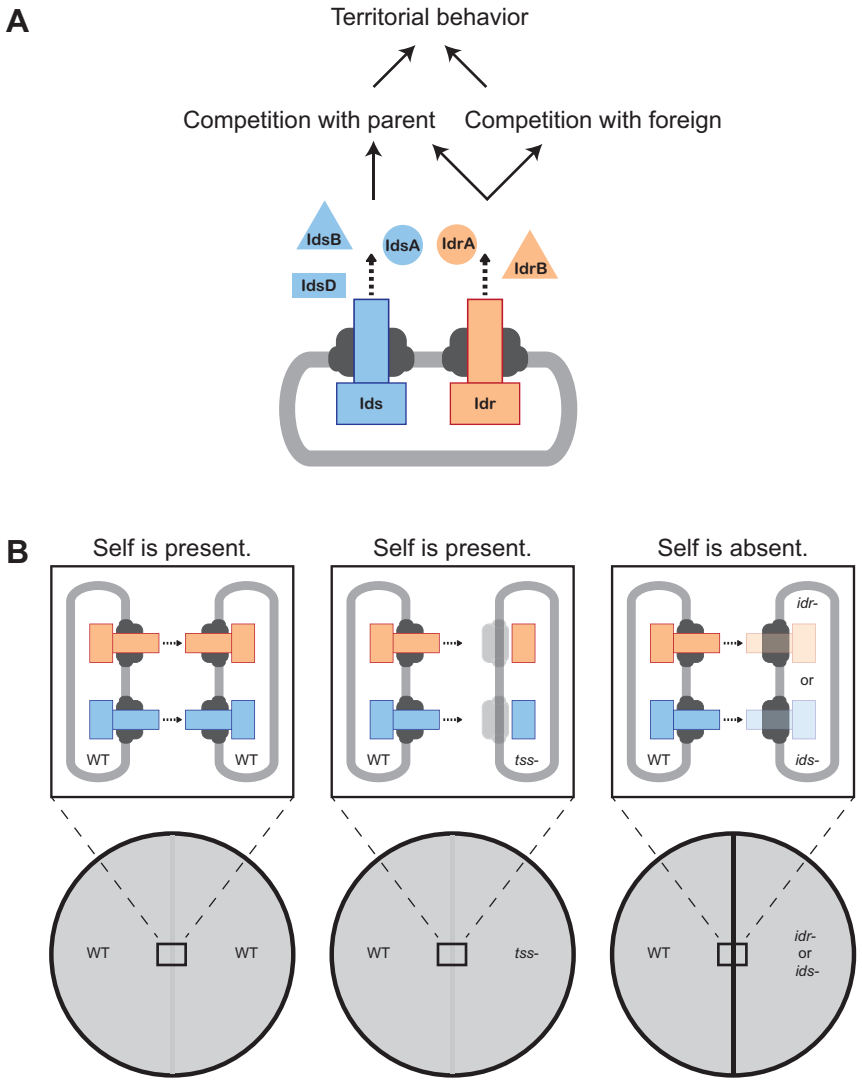

FIG 4 Model for Ids and Idr functional roles in self-recognition. (A) Functional flow chart for the roles of the Ids, Idr, and T6S proteins in self-recognition and territorial behaviors. A subset of Ids and Idr proteins are primarily exported via a shared T6S system (*tss*) and are necessary for competition on surfaces with the parent strain. Idr proteins are also needed for competition against foreign strains. (B) Our proposed model for self-recognition predicts that the combined actions of interactions between cognate Ids and Idr proteins between two neighboring cells result in the determination that self is present, ultimately resulting in the merging of two swarms. Expression of the self-recognition components within the cells is sufficient, though in wild-type strains, some of these components are exported from the cell by a T6S system. In contrast, absence of one or more of the Ids and Idr self-recognition systems leads to the determination that self is absent and ultimately to boundary formation.

system. Moreover, export of the Ids components is independent of the Idr components and vice versa.

DISCUSSION

Here we report the discovery of two additional gene clusters that together with the *ids* operon comprise a network of self-recognition genes (Fig. 4A). One locus, *idr*, encodes proteins necessary for merging with the parent BB2000 strain, while the other locus, *tss*, encodes a type VI secretion system that mediates export of Ids and Idr proteins. Significantly, we found that multiple Ids and Idr proteins are exported from the cell, including the self-identity determinant protein, IdsD.

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

Indeed, we demonstrate that the self-recognition capability of *P. mirabilis* provides a competitive advantage for the population specifically on surfaces. We did not observe similar advantages when the wild type was grown with self-recognition mutant strains in liquid (see the supplemental material). Growth on surfaces induces a broad developmental change in *P. mirabilis* where increased cell-cell contact yields increased population-wide coordination that is integral to migration and outward expansion of the swarm (reviewed in references 20 and 21). Perhaps the behavior of self-recognition is most beneficial in environments where social interactions are more frequent and thus potentially more impactful.

Our research supports a model in which *P. mirabilis* self-recognition involves the display of self-identity proteins that are likely interpreted via a direct physical interaction with other cells; this communication then yields a self-versus-nonself assessment that guides whether boundaries are formed between populations (Fig. 4B). Some self-identity proteins are likely displayed on or near the cell surface, as physical contact between cells is required for boundary formation (22). This extracellular exposure may serve to communicate a cell's identity represented by the self-identity determinant molecules IdsD and IdsE during interactions with neighboring cells (15, 16). Indeed, Ids and Idr proteins are transported out of the cell via the T6S system and either are trans-

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TABLE 1 Bacterial strains and plasmids

Strain or mutation	Genotype	Reference or source
Proteus mirabilis		
BB2000	Wild type	32
HI4320	Wild type	18, 40
Δ*ids*	Δ*ids*::Cm^r	15
Δ*ids* c. p*ids*$_{BB}$	Δ*ids*::Cm^r carrying a plasmid expressing the *ids* operon under the control of the *ids* upstream region	15
*idrB**	*idrB*::Tn-Cm^r	This study
*idrC**	*idrC*::Tn-Cm^r	This study
*idrD**	*idrD*::Tn-Cm^r	15
*tssA**	*tssA*::Tn-Cm^r	This study
*tssB**	*tssB*::Tn-Cm^r	This study
*tssG**	*tssG*::Tn-Cm^r	This study
*tssM**	*tssM*::Tn-Cm^r	This study
*tssN**	*tssN*::Tn-Cm^r	This study
BB2000 c. pKG101	Wild type carrying a plasmid with Kn^r and promoter-less *gfp*	16
*tssN** c. pLW100	*tssN*::Tn-Cm^r strain carrying a plasmid expressing *tssNOPQ* under the control of the *tssA* upstream region	This study
BB2000 c. pLW101	Wild type carrying a plasmid expressing IdsA-FLAG in which a FLAG was engineered to the C terminus of IdsA in the p*ids*$_{BB}$ vector	This study
Δ*ids* c. pLW101	Δ*ids*::Cm^r strain carrying a plasmid expressing IdsA-FLAG in which a FLAG was engineered to the C terminus of IdsA in the p*ids*$_{BB}$ vector	This study
*tssN** c. pLW101	*tssN**::Cm^r strain carrying a plasmid expressing IdsA-FLAG in which a FLAG was engineered to the C terminus of IdsA in the p*ids*$_{BB}$ vector	This study
Escherichia coli		
SM10λpir c. pUTmini-Tn*5*-Cm	Cm^r	41
S17-1λpir		41
XL10-Gold ultracompetent cells		Agilent Technologies, Santa Clara, CA

ported into the neighboring cell or localize on the cell surface (see the supplemental material). However, we have not yet found evidence for the transfer of self-recognition proteins into a neighboring cell.

We propose that boundary formation can result from the actions of a single population, which has queried on a cell-cell level whether the neighboring cell is self. For each population of *P. mirabilis* strain BB2000, "self" is defined by the combined actions of the Ids and Idr pathways. Self-recognition occurs when both the expected cognate Ids and Idr proteins are present in (or on) the neighboring cell, ultimately resulting in merging with the neighboring swarm (Fig. 4B). In contrast, we predict that boundary formation results from the absence of the cognate Ids and Idr self determinants in the neighboring cell (Fig. 4B). Both the Ids and Idr proteins likely initiate downstream signaling pathways that are altered depending on whether the interactions are with cognate or noncognate Ids and Idr proteins, respectively.

This two-part network for self-recognition appears analogous to aspects of the innate immune system and indeed has many parallels to the immune surveillance of natural killer (NK) cells. In current models for NK cell activity, the presence of self cells (i.e., of one's own organism) is conveyed by the combined detection of two surface receptors (an activation receptor ligand and class I major histocompatibility complex [MHC]), resulting in no killing of the self cell. In contrast, the absence of either receptor leads to the NK cell's determination of an absence of self and the subsequent killing of the nonself (or receptor-deficient) cell, as reviewed in reference 23. Intriguingly, these results in *P. mirabilis* further support the idea that cellular self-recognition is a behavior shared among organisms at many levels of biological complexity.

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

MATERIALS AND METHODS

Bacterial strains and media. All strains are listed in Table 1. *Escherichia coli* strains were maintained on LB agar and *Proteus mirabilis* strains were maintained on L swarm minus (LSW$^-$) agar (32). *P. mirabilis* was grown on CM55 blood agar base agar (Remel Inc., Lenexa, KS) for swarm colony growth. For broth cultures, all strains were grown in LB broth under aerobic conditions at 37°C. Antibiotics were used at the following concentrations: 15 μg/ml tetracycline (Tet); 100 μg/ml rifampin (Rif); 50 μg/ml kanamycin (Kn); 35 μg/ml chloramphenicol (Cm) for *E. coli* and 50 μg/ml Cm for *P. mirabilis*. All media contained antibiotics appropriate

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for selection or maintenance of plasmids. Strains carrying plasmids are so denoted with a "c."

Plasmid construction. The *tssN* (*icmF*) complementation plasmid pLW100 carries the last four genes of the *tss* gene cluster (*tssN* through *tssQ*) under the transcriptional control of the proposed promoter contained in the region immediately upstream of the *tss* gene cluster. This plasmid was constructed in two steps: the 1,200 bp upstream of *tssA* was amplified by PCR from the BB2000 genome and inserted into the pBBR1-*NheI* (15) plasmid using the Infusion HD system (Clontech Laboratories, Mountain View, CA); *tssN*, *tssO*, *tssP*, and *tssQ* were then PCR amplified and inserted into the above plasmid (with Infusion HD), resulting in pLW100. To construct pLW101, which is the plasmid encoding IdsA-FLAG, a FLAG epitope (N-DYKDDDDK-C) was inserted immediately before the *idsA* stop codon in the p*ids*$_{BB}$ plasmid (15) using QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). Plasmids were propagated in *E. coli* XL10-Gold and then conjugated into *P. mirabilis* via mating with *E. coli* S17-1λpir carrying the target plasmid as described earlier (15).

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

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

Mapping the transposon insertion sites. Arbitrary PCR was used to map the sites of the mini-Tn*5*-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 Tn*5*Ext and ARB6 for the first round and then oNS054 (5′ TTCACACAGGAAACAGCTATGACCGCA TTAAAATCTAGCGAGG 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′) by 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 or the Δ*ids*, *tssN**, or *idrB** mutant. Overnight cultures were normalized to an optical density at 600 nm (OD_{600}) of 0.1. Competing strains were mixed together in a 1:1 ratio, and 0.5 μl of each coculture was spotted onto the center of a CM55 agar plate. After incubation first at room temperature for 22 h and then at 37°C for 6 h, each swarm consisted of four swarm rings and was patched using a half-plate 48-prong device onto selective plates that could detect the marked BB2000 strain (LSW$^-$ Kn) and, when applicable, the mutant strain (LSW$^-$ Cm). Select swarms (i.e., BB2000 versus BB2000) were also plated nonselectively onto LSW$^-$ agar. To determine which strain was dominant in surface competitions, overnight cultures, normalized to OD_{600} of 1.0, of BB2000 were mixed at a 1:1 ratio with cultures of the Δ*ids*, *tssN**, or *idrB** strain. Mixed populations were inoculated onto CM55 agar using an inoculation needle; monocultures of the boundary indicator strains were inoculated approximately 1 cm away. After overnight incubation at 37°C, the presence or absence of boundaries between swarms was used to assess strain dominance at the leading edge. Dominance of BB2000 was assessed as the merger of mixed populations with a monoswarm of BB2000; dominance of the mutant was assessed as a merger with a monoswarm of itself or, in the case of the *tssN** strain, the Δ*ids* strain.

For competition between independently derived strains, the surface competition described above was repeated using the *P. mirabilis* wild-type strain HI4320 competed against BB2000 or the Δ*ids*, *tssN**, or *idrB** strain, with the modifications that a monoculture of HI4320 was used as the indicator strain on each plate instead of BB2000 and dominance of HI4320 was assessed as the merger of mixed populations with the HI4320 monoswarm. For all assays, unclear boundaries were recorded as "neither."

TCA precipitation. Overnight cultures were diluted to an OD_{600} of 0.1 in fresh LB+Kn and grown at 37°C with shaking to an OD_{600} of 3.5 to 4.5. For whole-cell extracts, 1 ml of culture was centrifuged, and the pellet was resuspended in 100 μl SDS-PAGE sample buffer. For supernatant samples, 30 ml of culture was clarified by centrifugation and filter sterilized (0.22-μm filters). The filtered supernatant was treated with trichloroacetic acid (10% final concentration) and incubated on ice for 30 min. Precipitated proteins were collected by centrifugation, washed twice with prechilled 100% acetone, dried, and resuspended in 100 μl 2× SDS-PAGE sample buffer.

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

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

Gel electrophoresis and Western blot. Protein samples were separated by gel electrophoresis using 15% Tris-Tricine gels and were either stained with Coomassie blue or transferred to nitrocellulose for Western blot analysis. Western blot membranes were probed with primary antibody (either 1:5,000 mouse anti-FLAG [Sigma-Aldrich, Allentown, PA], 1:1,000 mouse anti-sigma70 [Thermo Scientific, West Palm Beach, FL], or 1:1,000 rabbit anti-IdsB antiserum) for 1 h and with secondary antibody (1:5,000 goat anti-mouse-HRP [KPL, Gaithersburg, MD] or 1:5,000 goat anti-rabbit-HRP [KPL, Gaithersburg, MD]) for 1 h and visualized using Immun-Star HRP luminol/enhancer (Bio-Rad, Hercules, CA) and the ChemiDoc XRS system (Bio-Rad, Hercules, CA).

Nucleotide sequence accession number. The sequence for *tssA-Q* was deposited in NCBI under accession number BankIt1590180 BB2000 CP004022.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00374-13/-/DCSupplemental.

Figure S1, EPS file, 0.4 MB.
Figure S2, EPS file, 0.5 MB.
Figure S3, EPS file, 1.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.
Table S4, DOCX file, 0.1 MB.

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