Materials and Methods

Bacterial growth media and conditions. *Escherichia coli* was grown in modified L-broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of distilled water), solidified with 1.5% Bacto-agar as needed. *Proteus mirabilis* was also grown in modified L-broth. Agar media for *P. mirabilis* were Low Swarm Medium (LSW$^-$) (*S1*) for genetic manipulations and CM55 blood agar base (Oxoid Ltd., Hampshire, UK) for swarm experiments. Antibiotics were added at the following concentrations (per ml): 100 μg ampicillin (Ap); 100 μg rifampicin (Rf); 50 μg kanamycin (Kn); chloramphenicol (Cm) at 35 μg for *E. coli* and 150 μg for *P. mirabilis*. In all cases, growth was at 37°C.

Swarm studies. We used a method described by Senior (*S2*) with the modification that horse blood was omitted from the medium. Strains were inoculated from a plate colony using the end of a wooden dowel (2 mm diameter) at a spacing of approximately 2.25 cm onto the surface of a CM55 blood agar base plate without antibiotics. Boundary formation was examined 18 to 48 h after inoculation. Pictures of swarm plates were taken with a Pentax Optio W10 digital camera (Pentax Imaging Company, Westminster, CO). The contrast and brightness of images were equally increased across the entire raw image and then cropped to size in Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA).

Classification of recognition groups. Each recognition group was comprised of strains whose swarms merged with each other. Boundaries formed between swarms of strains in different recognition groups. Each strain was tested against every other strain to determine the recognition groups, and swarm plate assays were performed in duplicate. On each swarm plate, a standard boundary was included as a control. This was done because we have observed variation in the thickness and darkness of boundaries due to media and temperature conditions. For all experiments, the control boundary was either between BB2000 and the *idsA-F* deletion mutant or between HI4320 and a HI4320 strain carrying the BB2000 *idsA-F* gene cluster.

Experiments with mixed cultures. Equal numbers of cells from stationary phase cultures of BB2000 carrying pBBR1MCS-2 (Kn^R) and the *idsA-F* deletion mutant (Cm^R) were used as an inoculum (1%) in L-broth. The mixed culture was grown at 37°C for 24 h. Dilutions of the mixed culture were then spread on Kn and Cm plates to determine the ratio of BB2000 and *idsA-F* deletion mutant cells. Colonies were counted after 24 h at 37°C.

Microscopy. Pads of 0.5 ml CM55 agar mounted on glass microscope slides were inoculated with *P. mirabilis* at a spacing of 2.25 cm and incubated in a humidified chamber for 18 to 48 h. Images were acquired with a Nikon 80i epifluorescence microscope (Nikon Instruments Inc., Melville, NY) and a CoolSnap HQ camera (Photometrics, Tucson, AZ). The imaging system was operated with Metamorph 6.3r2 software (Molecular Devices, Sunnyvale, CA). Overlays of fluorescence and phase images were performed using the Metamorph software. The brightness and contrast of the phase and pseudocolor overlays images were increased equally across the entire raw images in Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA). Images were cropped equally for size constraints with the clipping mask function in Adobe Illustrator 11.0.0 (Adobe Systems Inc., San Jose, CA).

Transposon mutagenesis and screening for mutants. Mutagenesis of *P. mirabilis* BB2000 with mini*Tn*5 was performed as described elsewhere (*S1*). Mutants were collected from 36 independent biparental matings of *E. coli* S17-1λ*pir*[pUTmini*Tn*5-Cm] and BB2000 and were selected on Rf+Cm plates. One hundred colonies from each mating were picked for further studies, and a clonally purified 3,600 mutant library was arrayed in 96-well master plates (150 μl of L-broth with Rf and Cm per well). A 96-pin replicator was used to transfer 2 μl from each well in the master plates to the surfaces of swarm agar trays (75 mm X 116 mm Nunc Omnitray, Nalge Nunc International, Rochester, NY). After 18 to 24 h, each swarming colony was scored for boundaries with surrounding colonies.

Molecular techniques. To transform *P. mirabilis* BB2000, we used 16 μg of chromosomal DNA from the *P. mirabilis* transposon mutant and standard electroporation protocols (*S3*). We employed inverse PCR and DNA sequencing to identify the ΩCm^R insertion site in the transposon mutant (*S1, S3*). Sequencing of the *ids* loci from *P. mirabilis* strains BB2000 (*S1*), G151 (*S4*), CW977 (*S2*), CW677 (*S2*), I5/5 (*S2*) and S4/3 (*S2*) was performed by the DNA facility at the University of Iowa. Sequencing of the remaining constructs was performed by the DNA facility at the University of Iowa, by the DNA Sequencing and Gene Analysis Center in the Department of Pharmaceutics at the University of Washington and by SeqWright DNA Technology Services (Houston, TX). The GenBank accession numbers for the DNA sequence of the *ids* loci and flanking regions from strains BB2000, CW677, CW977, G151, I5/5 and S4/3 are EU635876, EU635877, EU635878, EU635879, EU635880 and EU635881, respectively. The sequence data for strain HI4320 were produced by and can be obtained from the *Proteus mirabilis* Sequencing Group at the Sanger Institute (*http://www.sanger.ac.uk/Projects/P_mirabilis/*) (*S5*) and were used with the written permission of the authors. The EMBL database accession numbers for the chromosome and plasmid of strain HI4320 are AM942759 and AM942760, respectively.

Strains and plasmids. *P. mirabilis* strains BB2000, HI4320 and HI4320[pBAC001], in which Green Fluorescent Protein (GFP) is constitutively expressed, were described previously (*S1, S6*). For microscopy, the *tac-fla-gfp* fragment from pBAC001 was cloned into pUTminiTn-Cm by using PCR amplification, and *E. coli* S17-1λ*pir* was transformed with the resulting construct. This strain was mated with *P. mirabilis* BB2000, and a Cm^R, Rf^R clone that recognized BB2000 as self and constitutively expressed GFP was selected. *P. mirabilis* strain BB2000 was transformed with pdsRed (Clontech, Mountain View, CA), and a Rf^R, Ap^R clone that recognized BB2000 as self and constitutively expressed dsRed was selected for use in epifluorescence microscopy. For construction of the *idsA-F* deletion (*Δids*) mutant, we used conjugation to introduce p*Δids*::ΩCm into strain BB2000. This suicide plasmid contains a Cm^R cassette flanked by 1.6 kilobase pairs (kbp) of DNA immediately upstream and 800 base pairs (bp) of DNA downstream of the *ids* locus. After conjugation with *E. coli* S17-1λ*pir* and selection for Rf+Cm resistant transconjugants, we screened for loss of the plasmid-encoded ampicillin resistance marker. One Ap^S colony was purified, and the *idsA-F* mutation was confirmed by DNA

sequencing. Strains BB2000, *Δids* and HI4320[pBAC001] were the recipients for conjugation with plasmids containing *ids* alleles.

All *ids* plasmids were constructed in pBBR1-*NheI*, derived from the broad host range vector pBBR1MCS-2 (*S7*) in which the *tac* promoter and multiple cloning sites were removed. The HI4320 *idsA-F* expression plasmid, named p*ids*$_{HI4320}$, has a 9.3-kbp fragment containing *idsA-F* and includes the 800-bp upstream DNA. The HI4320 *idsA-F* locus for cloning was generated as two PCR fragments by using chromosomal DNA from strain HI4320 as the template: an upstream 4.3-kbp NheI-PacI fragment and a 5-kbp downstream PacI-NgoMIV fragment. The PCR fragments were cloned into NheI and AgeI sites on the vector backbone to create p*ids*$_{HI4320}$, which retains the upstream NheI site but has lost both the synthetic NgoMIV site and the vector AgeI site. We similarly constructed the BB2000 *idsA-F* vector p*ids*$_{BB2000}$, except we used chromosomal DNA from strain BB2000 as the PCR template.

The plasmid containing the BB2000 *idsA-C* contained a 5-kbp region from 788-bp upstream of the predicted *idsA* translational start site to five bp downstream of the *idsC* stop codon. This region was PCR-amplified from BB2000 chromosomal DNA with engineered NheI and NgoMIV sites and cloned into vector pBBR1-*Nhe*I at the NheI and AgeI sites. The plasmid containing the BB2000 *idsD-F* was constructed by cloning two fragments amplified by PCR from BB2000 chromosomal DNA into pBBR1-*NheI*: one is a NheI-SalI fragment of the 788-bp region directly upstream of the *idsA* and the other is a SalI-NgoMIV fragment from 23 bp upstream of the predicted *idsD* start site to 199 bp downstream of the *idsF* stop codon.

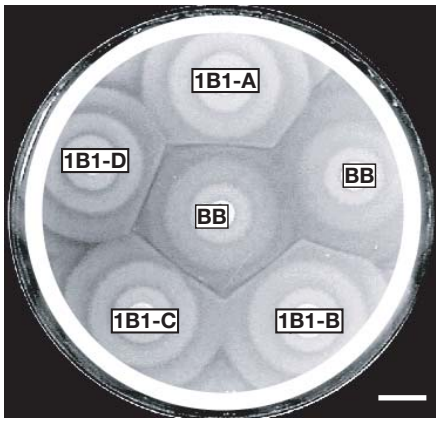
We constructed the following mutant alleles in p*ids*$_{BB2000}$. For disruption of *idsA*, p*ids*$_{BB2000}$ was digested with PmlI and HincII, which resulted in cleavage of an internal 315-bp fragment starting 137 bp from the *idsA* start. This yielded a linear 13-kbp fragment, which was gel-purified and ligated. For disruption of *idsB*, p*ids*$_{BB2000}$ was digested with AflII to remove a 711-bp fragment 152 bp downstream of the predicted *idsB* translational start. This yielded a 13-kbp linear fragment, which was gel-purified and ligated. For disruption of *idsC*, three stop codons (5'-taataatag-3') were engineered 73 bp from the predicted *idsC* translational start by using the QuikChange II XL Site-Directed Mutagenesis Kit per manufacturer's instructions (Stratagene, La Jolla, CA). Multiple clones were sequenced, and one with the correctly modified nucleotide sequence was selected. For disruption of *idsD*, a modified version of Splicing by Overlap Extension (*S8*) was performed to construct a PCR product with regions upstream and downstream of *idsD*: a 1.5-kbp region upstream of *idsD* including 37 bp of *idsD* and a 1.5-kbp region starting 35 bp upstream of the *idsD* stop codon and extending past *idsF*. This PCR product was cloned into p*ids*$_{BB2000}$ at the PacI site in *idsC* and the KpnI site in *idsF*. The disruption in *idsE* was constructed by replacing the 102-bp SacI-AgeI fragment internal to *idsE* with a 467-bp SacI-AgeI fragment from pBBR1MCS-2. This resulted in a frame-shift to a stop at codon 50. The disruption in *idsF* was constructed by inserting a 1.9-kbp *KpnI-Gm-GFP-KpnI* fragment from pPS858 (*S9*) into the KpnI site located 64 bp into the *idsF* open reading frame. The following mutant alleles were constructed in p*ids*$_{HI4320}$. The disruption in *idsD* was constructed by digesting p*ids*$_{HI4320}$ with EcoRI. This resulted in cleavage of an internal 1.8-kbp fragment, and the large 11-kbp fragment was isolated and

re-ligated. For the disruption in *idsEF*, we digested p*ids*$_{HI4320}$ with KpnI and AgeI, isolated the large 12-kbp fragment and ligated a 750-bp AgeI-KpnI fragment carrying a *gfp* marker obtained by PCR amplification.

All plasmids with disrupted alleles were used to transform *E. coli* DH5α and S17-1λ*pir*. *E. coli* S17-1λ*pir* was used for conjugation with *P. mirabilis* strains. Transconjugants were selected on Kn+Rf for BB2000, Kn+Cm for *Δids* and Kn+Ap for HI4320[pBAC001]. Multiple colonies were screened for resistance and confirmed by restriction enzyme digestion and sequencing. Single isolates were chosen for use.

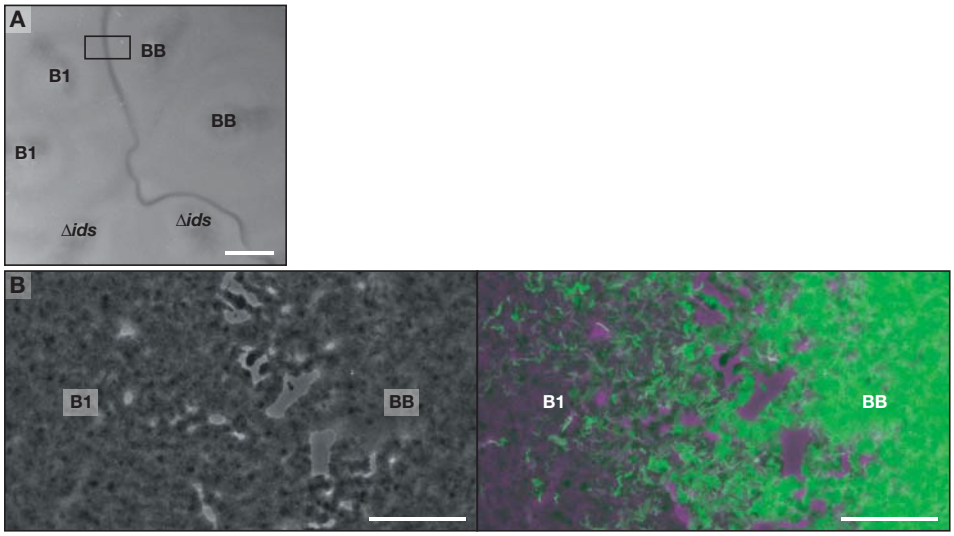
Bioinformatics. We used ClustalW2 for the alignments and to calculate the pair-wise identities of the polypeptides (*S10*). The graphical representations of the alignments were constructed using BOXSHADE (*http://www.ch.embnet.org/software/BOX_form.html*).

Figure S1



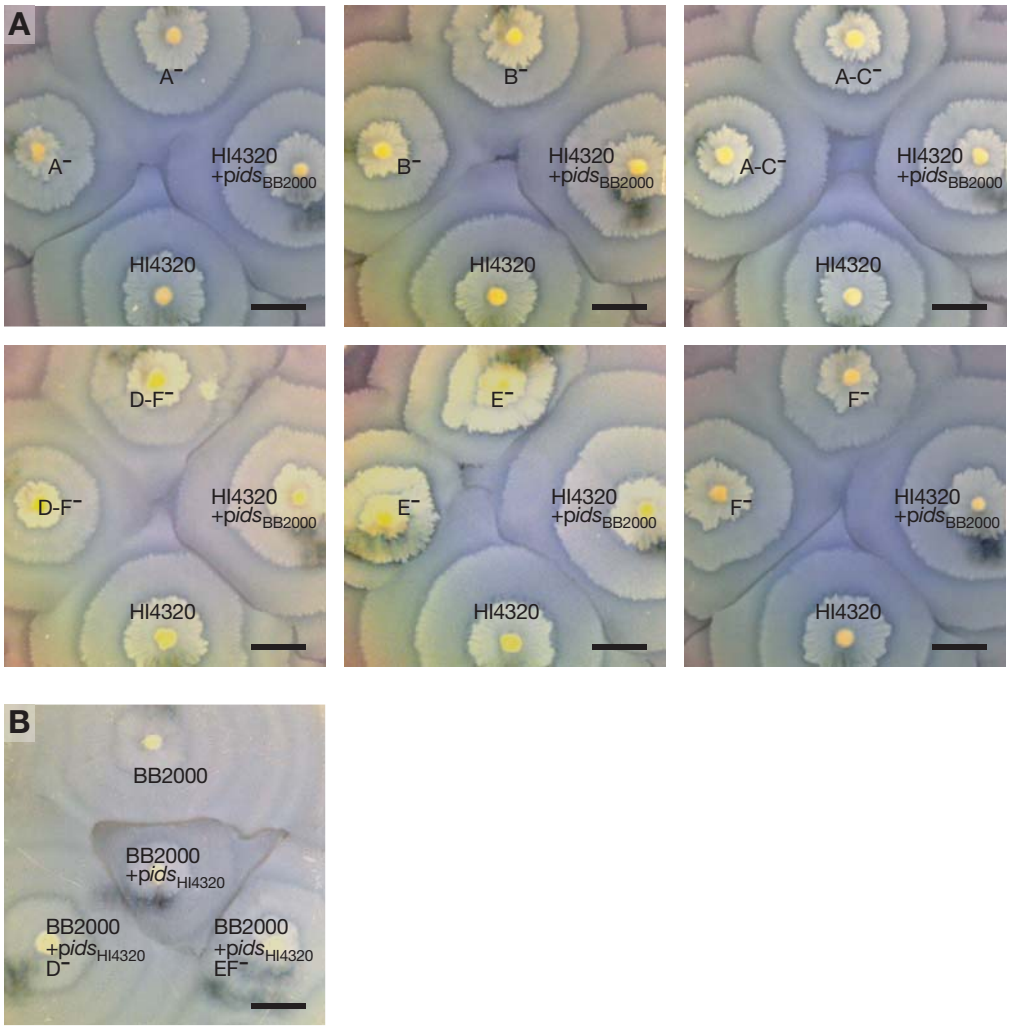
An agar plate with swarms from four independent homologous recombinants of the transposon insertion mutation into BB2000 (1B1-A, 1B1-B, 1B1-C and 1B1-D) surrounding swarms of the parent *P. mirabilis* strain BB2000 (BB). Boundaries formed between the 1B1 strains and BB, but not between BB and itself or between the recombinants. The bar is 1 cm.

Figure S2



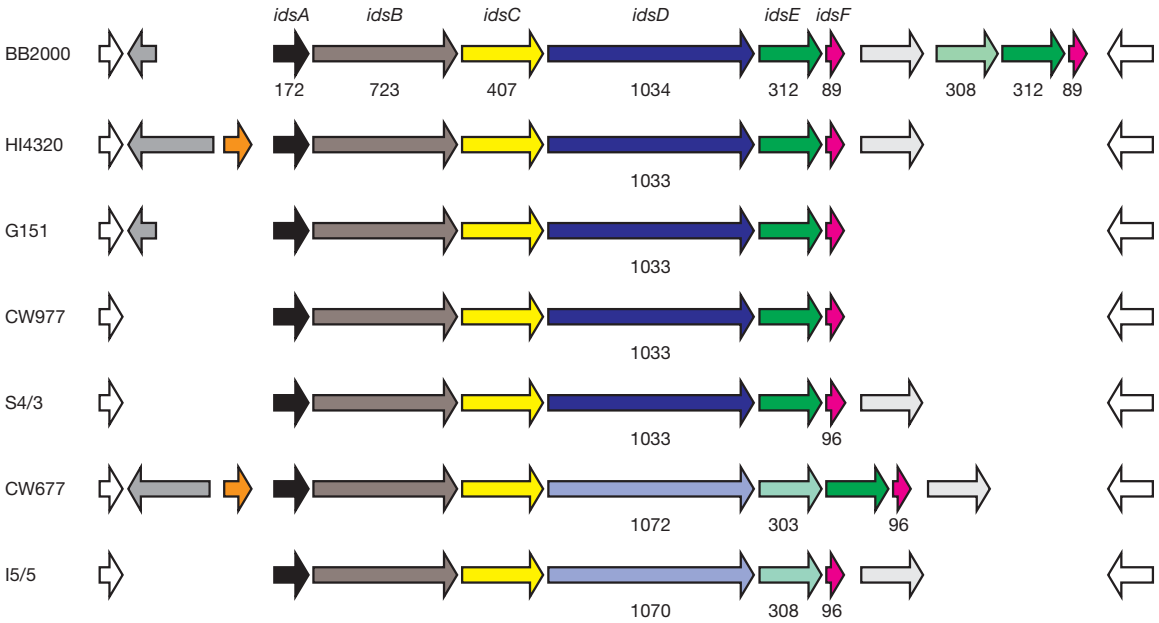
Boundary between wildtype *P. mirabilis* BB2000 and Ids mutants. (A) Section of an agar plate with pairs of the BB2000 parent (BB), the Ids transposon mutant (B1) and the *idsA-F* deletion mutant (*Δids*). This image is also shown in Gibbs et al. as Fig. 1D. The box indicates the boundary between the two swarms visualized in panel B. The bar is 1 cm. (B) Phase contrast micrograph (left) and pseudocolor overlay (right) of the boundary between a swarm of unlabeled B1 and a swarm of GFP-labeled BB (green). In the overlay image, the black coloring indicates areas occupied by unlabeled cells and the magenta coloring indicates regions devoid of cells. The bar is 50 μm.

Figure S3



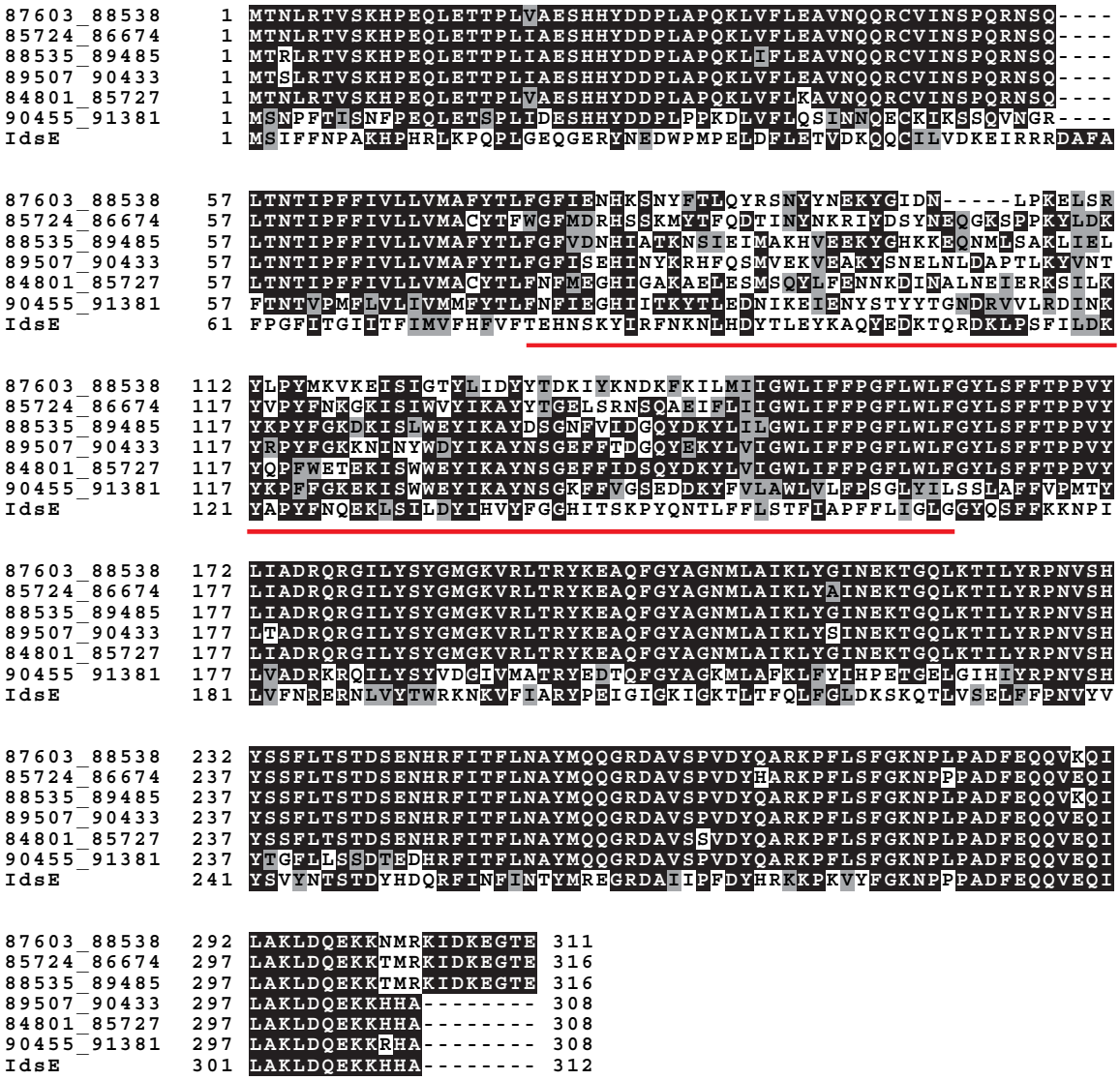
Boundary analysis of diploid *P. mirabilis* strains. (A) Sections of agar plates with *P. mirabilis* HI4320, diploid HI4320 strains carrying plasmids with the complete BB2000 *idsA-F* gene cluster (HI4320+p*ids*$_{BB2000}$), or diploid HI4320 strains carrying plasmids with the BB2000 *idsA-F* gene cluster with disruptions in *idsA* (A$^-$), *idsB* (B$^-$), *idsABC* (A-C$^-$), *idsDEF* (D-F$^-$), *idsE* (E$^-$) and *idsF* (F$^-$). (B) Section of an agar plate with diploid BB2000 strains carrying plasmids with the complete HI4320 *idsA-F* gene cluster (BB2000+*pids*$_{HI4320}$) or the HI4320 *idsA-F* gene cluster with disruptions in HI4320 *idsD* (BB2000+*pids*$_{HI4320}$*D*$^-$) and *idsEF* (BB2000+*pids*$_{HI4320}$EF$^-$). The bars are 1 cm.

Figure S4



Sequence analysis of the *ids* loci of BB2000, HI4320 and five other *P. mirabilis* isolates: G151, CW977, S4/3, CW677 and I5/5. All seven strains formed swarm boundaries with each other. The *ids* locus is found in the identical genomic region in all seven strains. It is downstream of an open reading frame (ORF) of 115 codons and upstream of a 225-codon ORF. The colors denote genes with significant sequence similarity, and the lighter shades of blue and green denote what appear to be divergent subfamilies based on the encoded polypeptide sequence. Sizes of the predicted ORFs are shown under the BB2000 map. The size for other strains is included if it differs from strain BB2000.

Figure S5



ClustalW2 alignment and BOXSHADE coloring of *idsE* from HI4320 with six polypeptides with sequence similarity to IdsE. These polypeptides are encoded in the HI4320 chromosome between base pairs 84801 to 91381. The red line underneath marks the variable region discussed in-text.

Supplementary References

S1. R. Belas, D. Erskine, D. Flaherty, *J Bacteriol* **173**, 6289 (1991).
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S4. M. A. Visalli, E. Murphy, S. J. Projan, P. A. Bradford, *Antimicrob Agents Chemother* **47**, 665 (2003).
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S10. R. Chenna *et al.*, *Nucleic Acids Res* **31**, 3497 (2003).