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Genetic Determinants of Self Identity and Social Recognition in Bacteria

Karine A. Gibbs,† Mark L. Urbanowski,‡ and E. Peter Greenberg

The bacterium Proteus mirabilis is capable of movement on solid surfaces by a type of motility called swarming. Boundaries form between swarming colonies of different P. mirabilis strains but not between colonies of a single strain. A fundamental requirement for boundary formation is the ability to discriminate between self and nonself. We have isolated mutants that form boundaries with their parent. The mutations map within a six-gene locus that we term ids for identification of self. Five of the genes in the ids locus are required for recognition of the parent strain as self. Three of the ids genes are interchangeable between strains, and two encode specific molecular identifiers.

A bout 60 years ago, different clinical isolates of the swarming bacterial species Proteus mirabilis were shown to form visually apparent boundaries between colonies growing on agar (1). By contrast, swarms of a single strain merge with each other (2) (Fig. 1A). This phenomenon is still used in diagnostic laboratories to type clinical isolates of P. mirabilis (3). Many clinical isolates of P. mirabilis secrete proteins called proteines that kill sensitive strains. An individual strain of P. mirabilis can be identified by a combination of the proteines it produces and the proteines to which it is sensitive (4,5). Boundaries form between swarms of strains differing in proteine production and sensitivity. However, some strains do not produce any proteines but still form boundaries, even with other non–proteine-producing strains. Thus, proteine production and sensitivity do not explain boundary formation. We sought to identify self versus nonself discrimination factors required for boundary formation by screening for and isolating mutants that recognize their parent as different from self. We chose P. mirabilis strain BB2000 as a model because it is genetically tractable (6). We used an agar plate assay to screen 3600 BB2000 mutants, generated by random transposon mutagenesis, in a format where each mutant swarm had two, three, or four adjacent neighbors (2). We found a single mutant that formed a boundary with every adjacent mutant, and we named the mutant phenotype “identification of self” (Ids) because mutant and parent swarms did not merge with each other. To show that the transposon insertion was responsible for the phenotype, we crossed the insertion in the Ids transposon mutant into the BB2000 parent by homologous recombination and isolated four recombinants, all of which formed boundaries with the parent but not with each other (Fig. S1).

Boundaries between strain BB2000 and the independent isolate H43320 contained individual cells of both strains at a low density as well as round bodies and debris. Cells of BB2000 and H43320 made contact with each other within the boundary, but we did not observe cells that penetrated the opposite swarm (Fig. 1B). In boundaries between swarms of the Ids transposon mutant and the BB2000 parent, we also observed a low density of cells, but round bodies and debris were not evident. Cells from the BB2000 parent swarm appeared to traverse the boundary and penetrate the Ids transposon mutant swarm (Fig. S2). During the merger of two swarms of the parent strain BB2000, cells from each swarm penetrated the opposite swarm without apparent hindrance (Fig. 1C).

References and Notes

19. Materials and methods are available as supporting material on Science Online.
36. We thank the European Synchrotron Radiation Facility (ESRF, Grenoble, France) for access to the beamlines and their staff for assistance during data collection. We are grateful to V. Rybin (European Molecular Biology Laboratory EMBO, Heidelberg, Germany) for initial ITC measurements; N. Hoffman for the original cpSRP clones; K. teKa, M. Groves, and U. Dürwag for their contributions; and A. Hendrickx for expert technical assistance. We thank M. Sattler (EMBL, Heidelberg) for stimulating discussions and NMR experiments and B. Dobberstein (ZMBH, University of Heidelberg, Germany) for helpful comments on the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SF638/1). The coordinates for the structures have been deposited in the Protein Data Bank under the accession codes 3D0E and 3DPE.

Supporting Online Material
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By using the sequenced genome of strain HI4320 (7), we found that the ids mutation mapped to codon 1030 of a 1033-codon open reading frame occurring between base pairs 3,282,912 to 3,286,013 and residing in a cluster of six genes (Fig. 2A). A homologous cluster was found by sequencing the parent BB2000 strain (2). We refer to the six-gene cluster as idsABCDEF for identification of self. We constructed an idsA-F deletion mutant of strain BB2000 and found that boundaries formed between swarms of the idsA-F deletion mutant and the BB2000 parent but not between the deletion mutant and the Ids transposon mutant (Fig. 1D). Complementation of the idsA-F deletion mutation with an idsA-F expression vector (which included the 800-bp region directly upstream of idsA) resulted in a transformant that merged with the BB2000 parent but formed boundaries with the deletion mutant (Fig. 1E). The complementation analysis confirmed that the idsA-F locus encodes self-recognition factors.

To assess individual ids genes, we introduced plasmids containing idsA-F gene clusters in which individual genes were disrupted into the idsA-F deletion mutant (2). We then tested all of the ids plasmid–carrying strains to determine whether they merged with each other or formed boundaries on swarm plates (Fig. 2B). We classified the constructs into recognition groups that were composed of strains whose swarms merged with each other but not with swarms of strains in different recognition groups (Fig. 2C).

An idsA-deficient strain merged with swarms of wild-type BB2000 but formed boundaries with the idsA-F deletion mutant (Fig. 2B and C). In contrast, idsB-, idsC-, idsD-, or idsE-deficient strains merged with the idsA-F deletion mutant but formed boundaries with wild-type BB2000. The idsF-deficient mutant likewise formed boundaries with wild-type BB2000 but had the additional property of forming boundaries with the idsA-F deletion mutant, and, in fact, swarms of the idsF-deficient mutant formed boundaries with swarms of any construct but itself. We conclude that idsA is not required for recognition of the BB2000 parent as self, but idsB, idsC, idsD, idsE, and idsF are required for self-recognition. The idsF gene appears to encode a recognition factor distinct in function from idsB-, idsC-, idsD-, or idsE-encoded factors, as indicated by the fact that idsF mutants merged only with themselves.

To further investigate the function of the ids genes in self-recognition, we introduced DNA containing either the complete BB2000 idsA-F gene cluster or a combination of disruptions in the BB2000 idsA-F gene cluster into wild-type HI4320 by conjugation to create transgenic diploids (2). The diploid H14320 strains partitioned into those that merged with wild-type HI4320 and those that formed boundaries with wild-type HI4320 (Fig. 2D and fig. S3). A diploid H14320 strain carrying the complete BB2000 idsA-F gene cluster formed boundaries with wild-type HI4320 but merged with swarms of diploid H14320 strains carrying the BB2000 idsA-F gene cluster with disruptions of idsA, idsB, idsC, or idsF (Fig. 2D).

In contrast, swarms of diploid H14320 strains carrying the BB2000 idsA-F gene cluster with disruptions in either idsD or idsE merged with wild-type HI4320 (Fig. 2D). Therefore, idsB, idsC, and idsF encode essential self-recognition functions, and the idsB, idsC, and idsF alleles can be complemented by alleles from a different strain. However, idsD and idsE are essential for self-recognition and appear to encode identity determinants.

To confirm that idsD and idsE encode identity determinants, we conjugated DNA containing the H14320 idsA-F gene cluster with gene disruptions in idsD and separately in idsEF into wild-type BB2000 (2). Swarms of both the idsD-deficient and idsEF-deficient diploid BB2000 strains merged with wild-type BB2000. However, a diploid BB2000

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Fig. 1. Images of swarm boundaries between different *P. mirabilis* strains. (A) Section of an agar plate with swarms of *P. mirabilis* strains HI4320 (HI) and BB2000 (BB). The boxes indicate intersections visualized in (B) and (C). (B) Microscopy showing (top) the boundary between a green fluorescent protein (GFP)–labeled HI swarm (green) and a red fluorescent protein (DsRed)–labeled BB swarm (red) and (bottom) a higher magnification of the boundary. The arrow indicates HI cells among BB cells in the boundary. (C) Microscopy showing (top) the merger of two BB swarms and (bottom) a higher magnification of the merger. The left BB swarm (green) expressed GFP, and the right BB swarm (red) expressed DsRed (2). (D) Section of an agar plate with swarms of BB, the Ids transposon mutant (B1), and the idsA-F deletion mutant (Δids). (E) Section of an agar plate with swarms of B1, Δids, BB, and two swarms of the idsA-F deletion mutant carrying an idsA-F expression vector (Δids+idsBB2000). The scale bars indicate 1 cm for (A), (D), and (E) and 10 μm for (B) and (C).
strain carrying the complete HI4320 idsA-F gene cluster formed boundaries with both idsD-deficient and idsEF-deficient diploid BB2000 strains (Fig. 2D). Thus, idsD and idsE encode identity determinants, which we refer to as molecular identifiers.

We note that a diploid BB2000 strain carrying the complete HI4320 idsA-F gene cluster formed boundaries with all other strains, including the diploid HI4320 strain carrying the complete BB2000 idsA-F gene cluster (Fig. 2D). Therefore, the idsA-F gene cluster is probably not the sole determinant of boundary formation between different strains. Consistent with the presence of additional unidentified boundaries, boundaries formed even in situations where one of the swarming strains did not carry any of the idsA-F genes (i.e., the idsA-F deletion mutant).

The idsA and idsB genes encode polypeptides with substantial sequence similarity to the conserved bacterial proteins Hep and VgrG, respectively. Recently, hep and vgrG were shown to form the first two genes in the type VI protein secretion system of Vibrio cholerae (8), and both hep and vgrG homologs occur in multiple copies in many bacterial species, including P. mirabilis (7–9). We have included idsA as part of the ids cluster even though it is not required for self-recognition because it is linked to idsB homologs in other bacteria and because it is possible that another hep homolog may be recruited to replace idsA in idsA-deficient P. mirabilis strains. The idsC, idsD, and idsE gene products do not show significant similarity to other known polypeptides. The idsF gene encodes a conserved hypothetical bacterial protein.

We sequenced the ids loci from five additional isolates of P. mirabilis: CW677, CW977, G151, IS5, and S43 (2). Swarms of the five strains formed boundaries with BB2000, HI4320, and each other. All strains had the six-gene ids locus except strain CW677, which had a seven-gene ids locus that contained an additional gene with similarity to idsE (fig. S4). In all strains, idsA, idsB, idsC, and idsF had over 96% identity with their homologs from the other strains (Fig. 3A). Both IdsD and IdsE could be separated into two distinct subfamilies with 30% pairwise identity. Within a single IdsD or IdsE subfamily, there was 97 to 99% pairwise identity across the majority of the sequence. However, within each subfamily, there was a C-terminal region in IdsD with only 72 to 84% pairwise identity and a similar region of only 32 to 80% pairwise identity in IdsE (between amino acids 80 and 169). The variable regions of idsD and idsE are reminiscent of alleles encoding antigenic variation in some bacterial pathogens (10).

The DNA immediately downstream of the idsA-F locus in strain BB2000 contains a gene coding for a polypeptide with sequence similarity to IdsF and two genes coding for polypeptides with similarity to IdsE (fig. S4). We do not know

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**Fig. 2.** Genetic analysis of the ids gene cluster. (A) The length of the encoded polypeptides in the six-gene ids cluster of strain BB2000 is shown underneath each gene. The lollipop marks the site of the transposon insertion in the ids transposon mutant. (B) Sections of agar plates with the BB2000 parent, the idsA-F deletion mutant (Δids), and the idsA-F deletion mutant carrying a plasmid-borne BB2000 idsA-F gene cluster with single-gene disruptions in idsA (A'), idsB (B'), idsC (C'), idsD (D'), idsE (E'), or idsF (F'). Scale bars, 1 cm. (C) Recognition groups of strains constructed in the BB2000 and Δids backgrounds. A subset of the strains is shown in (B). (D) Recognition groups of transgenic diploid derivatives of wild-type HI4320 and wild-type BB2000. Agar plates with swarms of representative strains are shown in fig. S3. For (C) and (D), a cell of each strain is represented by an oval in which the chromosomal and plasmid-borne ids gene clusters are drawn on the top and bottom, respectively. A white box denotes a gene disruption. The BB2000 and HI4320 ids gene clusters are in red and blue, respectively. Each strain was tested against every other strain to determine the recognition groups (2). Each recognition group was composed of strains whose swarms merged; boundaries formed between swarms of strains in different recognition groups.
whether there are additional IdsE or IdsF family members coded in the BB2000 genome, but the sequenced HI4320 genome contains a six-gene repeat between base pairs 84,801 and 91,381 that codes for polypeptides with similarity to IdsE (7) (fig. S5). It is possible that the putative IdsE homologs could act as additional molecular identifiers.

We have not yet succeeded in detecting the products of any of the ids genes in P. mirabilis cells, and so we do not know their cellular locations or how they might function to allow swarms to discriminate themselves from other encroaching swarms. It is unlikely that this is a toxin-antitoxin system because we do not see evidence of dead cells in the boundaries between the Ids transposon mutant and its parent (fig. S2) and because the ids-F deletion mutant and the BB2000 parent grew equally well in mixed cultures. When inoculated at a 1:1 ratio, the ratio of the parent to the ids-A-F deletion mutant in stationary phase remained 1:1. Instead, our data are consistent with a model for self-recognition in which idsD and idsE encode specific molecular identifiers of self. The idsB, idsC, and idsF products are devices necessary for self-nonself recognition, and the idsF product has a function distinct from those of idsB and idsC (Fig. 3B).

Self-recognition may play a role in maintaining clonal Proteus infections (11). It also seems likely that other species of bacteria have genes encoding self-recognition. In fact, there is a report of swarming boundary formation between strains of the opportunistic pathogen Pseudomonas aeruginosa (12). The P. mirabilis genetic model of swarm identity provides a simplified system to further examine the molecular mechanisms of self-nonself recognition.

Fig. 3. Organization and model of the ids gene cluster. (A) A plot showing the percent divergence of the encoded polypeptides in the idsABCDEF gene cluster among P. mirabilis strains BB2000, HI4320, CH977, G151, and S4/3. (B) A model for self-recognition. The idsB, idsC, idsD, idsE, and idsF genes are required for recognition of the BB2000 parent as self. IdsA is not required for self-recognition. IdsE has a function in self-nonself recognition that is distinct from that of IdsB and IdsC. The idsD and idsE genes encode specific molecular identifiers of self.

13. We thank B. Senior for generous sharing of many P. mirabilis strains and helpful comments on the swarm assay; R. Belas for providing strain BB2000; H. Mobley for providing strain HI4320; M. Visalli for providing strain G151; S. Chiquani, B. Duerkop, and A. Schafer for thoughtful scientific discussions; and the W. M. Keck Foundation for support. K.A.G. was supported by training grant AI55396 from NIH. The sequences of the ids loci and flanking regions from strains BB2000, CH977, CH977, G151, and S4/3 were deposited at GenBank, and the accession numbers are EU635876, EU635877, EU635878, EU635879, and EU635880, respectively.

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References
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Modulation of Gene Expression via Disruption of NF-kB Signaling by a Bacterial Small Molecule

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The control of innate immune responses through activation of the nuclear transcription factor NF-κB is essential for the elimination of invading microbial pathogens. We showed that the bacterial N-(3-oxo-dodecanoyl) homoserine lactone (C12) selectively impairs the regulation of NF-κB functions in activated mammalian cells. The consequence is specific repression of stimulus-mediated induction of NF-κB–responsive genes encoding inflammatory cytokines and other immune regulators. These findings uncover a strategy by which C12-producing opportunistic pathogens, such as Pseudomonas aeruginosa, attenuate the innate immune system to establish and maintain local persistent infection in humans, for example, in cystic fibrosis patients.

The innate immune system is activated in response to invading microbial pathogens through evolutionary conserved receptor-dependent mechanisms (1). For example, in mammals, the Toll-like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS) as a generic signal for an infection by Gram-negative bacteria (2). This in turn leads to the rapid activation of the nuclear transcription factor NF-κB and the expression of genes encoding proinflammatory cytokines, including tumor necrosis factor-α (TNF-α). Upon cellular stimulation with TNF through its receptors (TNFR), a positive signaling feedback loop in the NF-κB pathway prolongs LPS-induced gene expression (3, 4). NF-κB-dependent processes, in concert with other signaling cascades such as the p38 protein kinase pathway (5, 6), result in coordinated physiological responses that are critical for pathogen elimination (7–10). Despite the activation of the innate immune system, highly virulent bacteria are able to cause acute severe disease resulting from extensive bacteremia, and

References and Notes
2. Materials and methods are available as supporting material on Science Online.