Identification of non-flagellar genes involved in swarm cell differentiation using a *Bacillus thuringiensis* mini-Tn10 mutant library

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Swarming is a social phenomenon that enables motile bacteria to move co-ordinately over solid surfaces. The molecular basis regulating this process is not completely known and may vary among species. Insertional mutagenesis of a swarming-proficient *Bacillus thuringiensis* strain was performed, by use of the transposon mini-Tn10, to identify novel genetic determinants of swarming that are dispensable for flagellation, swimming motility, chemotaxis and active growth. Among the 67 non-swarming mutants obtained, six were selected that showed no defect in flagellar assembly and function, chemotaxis or growth rate. Sequence analysis of DNA flanking the transposon insertion led to the identification of previously uncharacterized genes that are involved in the development of swarming colonies by *B. thuringiensis* and that are highly conserved in all members of the *Bacillus cereus sensu lato* group. These genes encode non-flagellar proteins with putative activity as sarcosine oxidase, catalase-2, amino acid permease, ATP-binding cassette transporter, dGTP triphosphohydrolase and acetyltransferase. Functional analysis of two of the isolated mutants demonstrated that swarming differentiation depends on the intracellular levels of the osmoprotectant glycine betaine and on the quantity of synthesized phenazine secondary metabolites. The finding that proteins involved in diverse physiological processes have a role in swarming motility underlines the complexity of the molecular mechanisms governing this behaviour in *B. thuringiensis*.

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INTRODUCTION

Swarming motility is a social form of surface locomotion that is exhibited by a wide variety of Gram-negative and Gram-positive bacteria (for a review see Harshey, 2003 and Kaiser, 2007). This co-operative behaviour allows organisms to actively spread on suitably moist surfaces in the environment or on mucosal surfaces in the infected host. In several species, the ability to swarm is accompanied by a complex differentiation process leading swimmer cells to produce long, polyloid, hyperflagellated swarm cells (Fraser & Hughes, 1999; Harshey, 2003). These cells aggregate themselves in rafts and migrate over the surface in a highly co-ordinated manner that is dependent on multicellular interactions and cell-to-cell signalling (Rather, 2005). In swarming-proficient pathogens, swarm cells may exert a higher degree of virulence due to their enhanced ability to adhere to and colonize the host

mucosal surfaces (Allison *et al.*, 1992a; Belas & Colwell, 1982; Callegan *et al.*, 2006; Kirov *et al.*, 2004), as well as to secrete increased amounts of specific virulence factors (Allison *et al.*, 1992b; Ghelardi *et al.*, 2007; Macfarlane *et al.*, 2001).

Genome- and proteome-scale studies highlighted that swarming differentiation requires a wide range of cellular functions (Kearns *et al.*, 2004; Kim & Surette, 2004; Wang *et al.*, 2004). Nevertheless, the gene products specifically required for such a process are not completely known and may vary among species. Efficient assembly/function of flagella (Wang *et al.*, 2006) and chemotaxis (Mariconda *et al.*, 2006) are essential for swarm-cell differentiation in several swarming-proficient bacteria. In some species, biosynthesis of capsular polysaccharides, membrane lipopolysaccharides and biosurfactants plays a crucial role in promoting swarming or in facilitating bacterial migration across surfaces (for a review see Fraser & Hughes, 1999). In addition, cell density appears to be a critical cue for swarming, as demonstrated by the dependence of

Abbreviations: AHL, *N*-acylhomoserine lactone; T_{gen} , generation time; CI, chemotaxis index; PZ, phenazine.

swarm-cell differentiation on the quorum sensing mediated by extracellular *N*-acylhomoserine lactones (AHLs) in many Gram-negative bacteria (Fraser & Hughes, 1999).

We have previously described swarming motility in *Bacillus cereus* and *Bacillus thuringiensis* (Ghelardi *et al.*, 2002; Senesi *et al.*, 2002), two closely related bacterial species belonging to the *B. cereus sensu lato* complex (Rasko *et al.*, 2005). In these species, swarming requires differentiation of swimmer cells into elongated and hyperflagellated swarm cells and is a relatively widespread behaviour of natural isolates (Ghelardi *et al.*, 2007). In addition, the ability of these opportunistic human pathogens to swarm was proven to contribute to their pathogenicity, promoting invasive properties *in vivo* and leading to a notable increase in the secretion of virulence factors, in particular haemolysin BL (Callegan *et al.*, 2006; Ghelardi *et al.*, 2007; Senesi *et al.*, 2002).

In *B. cereus* and *B. thuringiensis* functional flagella and molecular components involved in both flagellum-mediated motility and chemotactic response are essential for swarming (Ghelardi *et al.*, 2002, 2007; Salvetti *et al.*, 2007; Senesi *et al.*, 2002). In this study, our aim was the identification of novel genes governing the transition from swimmer to swarm cells but dispensable for cell flagellation, motility, chemotaxis and active growth in *B. thuringiensis*.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *B. thuringiensis* strain 407 Cry[−] was used to produce the library of insertional mutants. *Escherichia coli* TOP10 was used as host for the construction of plasmids and cloning experiments and *E. coli* strain SCS110 for generating unmethylated plasmid DNA for *B. thuringiensis* transformation. *B. thuringiensis* strains were grown in tryptone-NaCl broth (TrB) (1% tryptone, 0.5% NaCl), brain heart infusion broth (BHI) or Luria-Bertani broth (LB) and usually incubated at 37 °C. Media were routinely solidified with 1% agar unless otherwise specified. Glucose (0.2%, w/v) was added to the media when required. *E. coli* strains were grown in LB medium at 37 °C. Antibiotics used for bacterial selection included ampicillin (100 µg ml^{−1}) or spectinomycin (60 µg ml^{−1}) for *E. coli* and spectinomycin (250 µg ml^{−1}) or erythromycin (25 µg ml^{−1}) for *B. thuringiensis*. Growth curves of *B. thuringiensis* strains were analysed in LB medium and the generation time (T_{gen}) was calculated as follows: $T_{\text{gen}} = t/[3.3 \log(b/B)]$, where t is the time interval in minutes, b the number of bacteria at the end of the time interval, and B the number of bacteria at the beginning of the time interval. The biochemical profile of *B. thuringiensis* strains was determined by using the API 50-CHB and API 20E strips tests (bioMérieux) accordingly to the manufacturer's instructions; test results were read after 24 h and 48 h with the ATB Plus software (bioMérieux).

Motility and chemotaxis assays. Phenotypic assays for swimming were performed on TrB fortified with 0.25% granulated agar (TrM) and chemotaxis assays on TrM supplemented with 2 mM mannitol, as previously described (Salvetti *et al.*, 2007). Each chemotaxis assay was repeated three times on separate days and the chemotaxis index (CI) for each strain was calculated as follows: $CI = (D_a - D_c)/D_c$, where

D_a is the mean of growth halo diameters recorded on TrM plates supplemented with mannitol and D_c the mean of those measured in TrM without the attractant.

The ability to swarm was tested on BHI, LB, TrB and the same media supplemented with glucose (BHIG, LBG, TrBG) at various agar concentrations (0.8%, 1.0%, 1.2%, 1.4%). Plates were centrally inoculated by spotting 0.5 µl of an overnight culture (approx. 2×10^8 cells ml^{−1}) and incubated at 37 °C in a humidified chamber. The swarming patterns and the presence of differentiated swarm cells were evaluated at 6, 12, 18, 24 and 48 h post-inoculation. Formation of swarm cells was assessed by analysing cell length and the amount of cell-surface flagella, by phase-contrast microscopy of bacteria stained with a solution containing 10% (w/v) crystal violet (Salvetti *et al.*, 2007) or flagellar staining for light microscopy (Harshey & Matsuyama, 1994), respectively.

Mutagenesis. Transposition of the mini-Tn10 into the *B. thuringiensis* 407 Cry[−] genome was performed using the thermosensitive plasmid pIC333 (Steinmetz & Richter, 1994) as a delivery vector. About 1 µg of pIC333 was used to transform strain 407 Cry[−] by electroporation. Transformants were selected at 28 °C on LB plates containing both spectinomycin and erythromycin. Antibiotic-resistant clones were pooled and cultured at 28 °C in LB broth containing spectinomycin for 3 h. Cells were diluted (1:100) and grown in LB broth at 40 °C (non-permissive temperature for the replication of the plasmid) for 15 generations. Appropriate dilutions of the culture were then spread on LB agar containing spectinomycin and incubated overnight at 37 °C. Following growth, bacteria were analysed for resistance to erythromycin; only erythromycin-sensitive clones were collected to produce the library of insertional mutants.

Identifying sites of transposon insertion. To identify the region of mini-Tn10 insertion, chromosomal DNA was extracted from selected mutants, as previously described (Senesi *et al.*, 2002), digested with *Eco*RI or *Hind*III (restriction sites for these enzymes are not present in the mini-Tn10) and subjected to ligation. As the mini-Tn10 contains an *E. coli* origin of replication, the ligation mixture was used to transform *E. coli* strain TOP10, and spectinomycin-resistant clones were selected. Plasmid DNA was extracted from the *E. coli* transformants by the NucleoSpin Plasmid Purification kit (Machery-Nagel) and sequenced using primers E1 and E3 (Table 2), matching the ends of the mini-Tn10, as described by Gominet *et al.* (2001). DNA sequencing was performed by the ALFexpress AutoRead Sequencing kit (Pharmacia Biotech) in the ALFexpress DNA sequencer (Pharmacia) and all ORFs longer than 100 bp were examined.

Southern blot analysis was performed as described elsewhere (Senesi *et al.*, 2002), using Hybond-NX membranes (Amersham Biosciences) and ³²P-dCTP-labelled DNA probes.

Sequence analysis. The DNA sequences surrounding the sites of mini-Tn10 insertion were analysed by using the software Pathema-Bacillus (<http://pathema.jcvi.org/cgi-bin/Bacillus/PathemaHomePage.cgi>). This resource was chosen since it contains an in-depth curatorial analysis of micro-organisms belonging to the *B. cereus* group and offers single-genome and comparative multi-genome analysis for these bacteria. The deduced amino acid sequences were compared against all *Bacillus* peptide files running WU-BLAST 2.0 available in Pathema-Bacillus or analysed in the GenBank, EMBL and Swiss-Prot databases by using the BLASTX, BLASTP or PSI-BLAST network service at the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1997). To predict the presence of transmembrane helices and topology of putative proteins, the amino acid sequences were analysed using the programs TMPred and PredictProgram at the ExPASy proteomics server of the Swiss Institute of Bioinformatics (<http://expasy.org>).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic	Source or reference
<i>Bacillus thuringiensis</i>		
407 Cry ⁻	Swarming-proficient wild-type strain	Lereclus <i>et al.</i> (1989)
MP10	Swarming-defective derivative of 407 Cry ⁻	This study
MP11	Swarming-defective derivative of 407 Cry ⁻	This study
MP12	Swarming-defective derivative of 407 Cry ⁻	This study
MP13	Swarming-defective derivative of 407 Cry ⁻	This study
MP14	Swarming-defective derivative of 407 Cry ⁻	This study
MP15	Swarming-defective derivative of 407 Cry ⁻	This study
MP16	Derivative of MP10, containing pHT2787	This study
MP17	Derivative of MP11, containing pHT2969	This study
MP18	Derivative of MP12, containing pHT0713	This study
MP19	Derivative of MP13, containing pHT3576	This study
MP20	Derivative of MP14, containing pHT5364	This study
MP21	Derivative of MP15, containing pHT3423	This study
MP22	Derivative of MP15, containing pHT3423-3422	This study
<i>Escherichia coli</i>		
SCS110	<i>rpsL</i> (Str ^r) <i>thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44</i> Δ (<i>lac-proAB</i>) [<i>F'</i> <i>traD36 proAB lacZ</i> Δ M15]	Stratagene
TOP10	<i>F</i> ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> AM15 Δ <i>lacX74 deoR recA1 endA1 ara</i> Δ 139 Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL</i> (Str ^r) <i>nupG</i>	Invitrogen
Plasmids		
pIC333	Thermosensitive Gram-positive origin of replication; 2.4 kb mini-Tn10 containing a ColE1 origin of replication and an <i>Sp</i> ^c gene; Tn10 transposase gene; Em ^r	Steinmetz & Richter (1994)
pHT304	Expression vector with <i>lacZ</i> promoter; Gram-positive origin of replication, <i>ori E. coli</i> ; Em ^r Ap ^r	Arantes & Lereclus (1991)
pHT2787	Derivative of pHT304, containing the BT2787 gene amplified from <i>B. thuringiensis</i> 407 Cry ⁻	This study
pHT2969	Derivative of pHT304, containing the BT2969 gene amplified from <i>B. thuringiensis</i> 407 Cry ⁻	This study
pHT0713	Derivative of pHT304, containing the BT0713 gene amplified from <i>B. thuringiensis</i> 407 Cry ⁻	This study
pHT3576	Derivative of pHT304, containing the BT3576 gene amplified from <i>B. thuringiensis</i> 407 Cry ⁻	This study
pHT5364	Derivative of pHT304, containing the BT5364 gene amplified from <i>B. thuringiensis</i> 407 Cry ⁻	This study
pHT3423	Derivative of pHT304, containing the BT3423 gene amplified from <i>B. thuringiensis</i> 407 Cry ⁻	This study
pHT3423-3422	Derivative of pHT304, containing the BT3423/BT3422 gene region amplified from <i>B. thuringiensis</i> 407 Cry ⁻ with the primer pair BT3423U/BT3422L	This study

Complementation. For complementation experiments, the genes disrupted by the transposon were amplified by PCR from *B. thuringiensis* 407 Cry⁻ by using primers designed on the basis of *B. thuringiensis* *konkukian* DNA sequence (Table 2). Each PCR product was purified, digested (for the restriction enzyme used, see Table 2), and ligated into the expression vector pHT304 (Arantes & Lereclus, 1991). The mini-Tn10 mutants were transformed with the recombinant plasmids (Table 1) and selected for erythromycin resistance.

Sarcosine oxidase activity. Sarcosine oxidase activity was assayed in crude extracts of bacterial cultures that were normalized to the same OD₆₀₀, as described by Koyama *et al.* (1991), using 0.2 M sarcosine or 0.2 M N,N-dimethylglycine (Sigma Aldrich) as the substrate. Each reaction mixture was incubated at 37 °C for 10 min. One unit (U) of the enzyme was defined as the amount of enzyme which liberated 1 µmol formaldehyde per minute.

Phenazine quantification. Phenazines (PZs) were quantified essentially as described by Maddula *et al.* (2008). Briefly, bacteria were grown in 5 ml BHIG broth at 30 °C to the late-exponential phase and cell-free culture supernatants were acidified to pH 2 with concentrated HCl. Total PZs were extracted with benzene, dissolved in 0.1 M NaOH, quantified via absorbance at 367 nm and expressed

as A₃₆₇ for 10¹⁰ bacterial cells. PZs were also quantified after growth under swarming condition by growing bacteria on Anopore membranes (0.2 µm pore size) of 10 mm cell culture inserts (Nalge Nunc International) in BHIG as previously described (Ghelardi *et al.*, 2007). After 48 h incubation at 30 °C, the number of c.f.u. on the membranes was counted, and PZs were quantified as described above.

Statistical analysis. Statistical analysis was performed using the GraphPad InStat Software. A *P*-value <0.05 was considered statistically significant. Values were expressed as the mean $\pm$ SD from five independent experiments.

RESULTS AND DISCUSSION

Phenotype of *B. thuringiensis* 407 Cry⁻ swarming colonies on different solid media

Swarming in *Bacillus* spp. is a recently described phenomenon. In these species, swarming motility occurs on rich media at a wide range of agar concentrations and temperatures (Ghelardi *et al.*, 2002; Senesi *et al.*, 2002,

Table 2. Primers used for PCR amplification

Primer	Sequence (5'–3')*	Target/base pair coordinates†	Restriction site
BT2787U	GATAAGCTTATAATACACTT	BT2787, from –145 to –125	<i>Hind</i> III
BT2787L	TCCGGATCCTACATCATGAC	BT2787, from +1364 to +1344	<i>Bam</i> HI
BT2969U	CTAGCATGCTGTTACTAT	BT2969, from –102 to –84	<i>Sph</i> I
BT2969L	ATTGGATCCGTTGCAATG	BT2969, from +1570 to +1552	<i>Bam</i> HI
BT0713U	CGTCTGCAGTTTACAGA	BT0713, from –71 to –53	<i>Pst</i> I
BT0713L	GATGGATCCTTTTAGTTG	BT0713, from +1731 to +1713	<i>Bam</i> HI
BT3576U	AATAAGCTTTAGAATAT	BT3576, from –143 to –126	<i>Hind</i> III
BT3576L	TTAGCATGCGAAACGGA	BT3576, from +1850 to +1833	<i>Sph</i> I
BT5364U	AGCAAGCTTTCACATT	BT5364, from –141 to –125	<i>Hind</i> III
BT5364L	AAGCTGCAGAACTGGTG	BT5364, from +1447 to +1429	<i>Pst</i> I
BT3423U	TGAAAGCTTCTGAAGGAG	BT3423, from –103 to –85	<i>Hind</i> III
BT3423L	TATGGATCCTACTTCTGA	BT3423, from +965 to +947	<i>Bam</i> HI
BT3422L	TTTGGATCCCGCTATTTCG	BT3422, from +984 to +966	<i>Bam</i> HI
E1	CGTTGGCCGATTCAATATGC	Mini-Tn10	None
E3	CGATATTCACGGTTTACCCAC	Mini-Tn10	None

*Restriction site is underlined.

†Numbers refer to nucleotide position in the indicated gene, relative to the putative translational initiation site.

2004). Nevertheless, the possibility of predicting swarming differentiation by measuring colony diameter or observing the colony appearance was proven to be inapplicable for strains belonging to the *B. cereus sensu lato* group (Ghelardi *et al.*, 2007; Hsueh *et al.*, 2007). The ability to swarm by *B. cereus* and *B. thuringiensis* can only be ascertained by visualizing the presence of elongated and hyperflagellated cells inside the developing colony (Ghelardi *et al.*, 2002; Senesi *et al.*, 2002). To facilitate the screening of swarming-defective derivatives of strain 407 Cry[–], the wild-type strain was inoculated on a variety of rich media (BHI, BHIG, LB, LBG, TrB, TrBG) containing different agar concentrations (0.8 %; 1.0 %; 1.2 %; 1.4 %) and analysed for colony type and swarm cells after incubation at 37 °C for 6, 12, 18, 24 and 48 h. Long and extensively flagellated swarm cells were visualized after growth on all tested media at the different time points examined (data not shown). However, the colony produced on BHIG fortified with 1.4 % granulated agar after 48 h was the widest we obtained and was characterized by concentric rings due to alternate cycles of swarming migration and consolidation phases (Fig. 1), as described for swarming by *Proteus mirabilis* (Douglas & Bisset, 1976). Therefore, this culture condition was chosen and applied throughout the study for the screening of swarming-defective mutants of strain 407 Cry[–].

Production, isolation and characterization of non-swarming mutants

With the aim of identifying genes required for swarming by *B. thuringiensis*, insertional mutagenesis of strain 407 Cry[–] was carried out using the transposon mini-Tn10. Approximately 5000 spectinomycin-resistant and erythromycin-sensitive mutants were isolated and screened for

swarming defects. On inspection of the mutant library, 67 strains generated a colony that was clearly different from that produced by the wild-type. All these strains exhibited partially reduced colony spreading and lacked the characteristic colony organization in concentric rings. Each of the 67 selected strains was analysed for swimming motility on TrM in comparison with strain 407 Cry[–], to exclude all the mutants showing swimming defects. Twenty-eight strains did not generate any migration halo (data not shown); the high number of mutants displaying a defective swimming phenotype probably reflected the high number of genes required for flagella assembly and/or functionality in which the transposon insertion could have occurred. Among the remaining mutants ($n=39$), 14 showed growth defects in liquid medium (prolonged lag phase and/or increased generation time) and were not analysed further. Swimming-proficient and normally growing strains ($n=25$) were inoculated on TrM plates containing mannitol to evaluate their ability to undergo chemotaxis. Since the CI value of strain 407 Cry[–] was 0.94, all strains displaying CI values lower than 0.5 were excluded from the study. On the basis of the CI value of each mutant, eight strains were selected that did not show evident chemotactic defects. To evaluate whether the transposon insertion caused metabolic alterations, the biochemical profile of each mutant was analysed. No difference was found in the ability to use carbohydrates or amino acids for growth by using the API 50-CHB and API 20E strip tests.

Among the eight mutants, some strains were subsequently demonstrated to contain identical transposon insertions and hence were likely to be siblings. Leaving these aside, our mutant collection consisted of six strains which showed: (I) overlapping growth curves (T_{gen} ranging from 22.06 ± 2.4 to 27.91 ± 2.8 min) and biochemical profile;

Fig. 1. Phenotype of *B. thuringiensis* 407 Cry⁻ swarming colonies on different media. The strain was centrally inoculated on BHI, LB, and TrB fortified with 1.4% granulated agar with or without 0.2% glucose. After 48 h incubation at 37 °C, plates were photographed against a black background. Black bars in the upper images indicate the concentric rings inside the swarming colony. White scale bars indicate 1 cm.

(II) similar proficiency for swimming motility and chemotaxis (Table 3); and (III) similar colony morphotype on BHIG fortified with 1.4% agar, but remarkably different from that developed by the wild-type strain (Fig. 2). Finally, the colonies produced by the selected mutants were analysed for the presence of differentiated swarm cells. None of the strains were able to produce hyperflagellated and elongated cells, and only oligoflagellated swimmer cells were viewed during colony development (Table 3, Fig. 2).

Identification of the genes disrupted in the non-swarming mutants

As no *Hind*III or *Eco*RI restriction sites are present in the mini-Tn10, the six swarming-defective mutants were analysed for the presence of the transposon by Southern hybridization of *Hind*III or *Eco*RI chromosomal DNA digestions. A single reactive band was detected for all strains, indicating that each mutant carried a single genomic insertion (data not shown). The DNA flanking the transposon was isolated and cloned into *E. coli* (see Methods) and the recombinant plasmids were used as templates to determine the nucleotide sequence of the chromosomal DNA adjacent to the mini-Tn10 insertion site. The mini-Tn10 generates a 9 bp duplication at the site of insertion, which served as a tag to identify the disrupted gene (Table 4).

The sequences obtained for each strain were submitted to the Pathema-Bacillus software; the nomenclature of *B. thuringiensis konkukian* genes was adopted to indicate the interrupted genes, as no complete chromosomal sequence is available for strain 407 Cry⁻ of *B. thuringiensis* (Table 4). This search revealed that the transposon had inactivated non-flagellar genes highly conserved in all completely sequenced members of the *B. cereus sensu lato* group. Fig. 3 shows the gene organization of the regions comprising the disrupted genes on the *B. thuringiensis konkukian* genome. The polar effects of the mini-Tn10 insertion were evaluated

Table 3. Motility of *B. thuringiensis* strains in liquid and solid environment

Strain	Liquid environment			Solid surface		
	MOT*	CI†	Max. cell length (µm)	Hyper-FLA‡	Max. cell length (µm)	SW§
407 Cry ⁻	+	0.94	4.0	+	13.2	+
MP10	+	0.78	4.0	–	6.0	–
MP11	+	0.66	3.8	–	5.1	–
MP12	+	0.85	4.1	–	4.0	–
MP13	+	1.20	4.0	–	5.9	–
MP14	+	1.40	3.9	–	4.0	–
MP15	+	1.00	4.0	–	4.2	–

*Swimming motility was evaluated on tryptone-NaCl plates containing 0.25% agar (TrM).

†Chemotaxis index was calculated as described in Methods.

‡Cell hyperflagellation was defined as a >3-fold increase in flagellation from liquid to solid medium.

§Swarming motility was evaluated by the presence of hyperflagellated and elongated cells.

Fig. 2. Analysis of swarming behaviour of *B. thuringiensis* strains. *B. thuringiensis* 407 Cry[−] and its derivative mutants were centrally inoculated on BHIG fortified with 1.4 % granulated agar and incubated for 48 h at 37 °C. Insets show the phenotype of cells collected from the colony rim.

by introducing into each mutant a wild-type copy of the interrupted gene by the plasmid pHT304, thus producing strains MP16–MP21 (Table 1). Only strain MP21 did not restore a swarming phenotype, thus indicating that transposition TnΩ4245 could generate polar effects on the expression of the downstream gene (Table 4, Fig. 3).

BT2787 encodes monomeric sarcosine oxidase

The transposition TnΩ63 occurred in a BT2787 homologous gene. BT2787 had not been previously characterized in the genus *Bacillus*. However, it is predicted to encode the monomeric form (EC 1.5.99.2) or the beta subunit (EC 1.5.99.1) of multimeric sarcosine oxidase, which are involved in the glycine-betaine degradation pathway and show strong sequence similarity (Chen *et al.*, 2006). Monomeric sarcosine oxidase catalyses the oxidation of *N,N*-dimethylglycine into sarcosine (*N*-methylglycine), which is converted into glycine by the multimeric enzyme. Oxidation of both substrates generates formaldehyde,

whose production is used to quantify the enzyme activity. To establish which enzyme was defective in strain MP10, the enzyme activity was assayed in bacterial crude extracts using sarcosine or *N,N*-dimethylglycine as the substrate. No defect in the conversion of sarcosine into glycine was found, the enzyme activity being 0.28 ± 0.07 and 0.34 ± 0.06 U ml^{−1} for the mutant and the wild-type strain, respectively ($P=0.18$). This result suggested that the mini-Tn10 insertion had occurred in the gene encoding the monomeric form of sarcosine oxidase. Indeed, this enzymic activity was only found in strain *B. thuringiensis* 407 Cry[−] (0.030 ± 0.006 U ml^{−1}) and no formaldehyde was ever generated by the mutant crude extract using *N,N*-dimethylglycine as the substrate. Since high levels of intracellular glycine betaine are obtained by inhibition of the enzymes involved in its degradation pathway (Lucht & Bremer, 1994; Smith *et al.*, 1988), the mutation in BT2787 could lead to an increase in the cytoplasmic levels of this compound. Glycine betaine is a very efficient osmolyte that accumulates intracellularly in response to sudden increases

Table 4. Transposon insertion sites that abolish swarming differentiation

Strain	Transposon insertion	Transposon insertion site	Polar effect of transposon insertion*	Homologous gene†	Identity (%)‡	Putative function
MP10	TnΩ63	GGTTGCGCT	No	BT2787	86	Sarcosine oxidase
MP11	TnΩ940	TACGAAGTT	No	BT2969	87	Catalase-2
MP12	TnΩ962	CATTAAGCT	No	BT0713	83	Amino acid permease
MP13	TnΩ1851	TGCTGAGTA	No	BT3576	86	ABC transporter, substrate-binding protein
MP14	TnΩ4040	GATCAAGTA	No	BT5364	90	dGTP triphosphohydrolase
MP15	TnΩ4245	TACTTGGAT	Yes	BT3423	90	Acetyltransferase

*Deduced by trans-complementation experiments performed as described in Methods.

†Nomenclature of the homologous genes in *B. thuringiensis konkukian*.

‡Percentage similarity values of the *B. thuringiensis* 407 Cry[−] ORFs to those of *B. thuringiensis konkukian*.

Fig. 3. Chromosome maps of *B. thuringiensis konkukian* indicating sites of transposon insertions that impaired swarming in *B. thuringiensis* 407 Cry⁻. (a) MP10; (b) MP11; (c) MP12; (d) MP13; (e) MP14; (f) MP15. Coding regions are represented as horizontal arrows. Each insertional mutation is represented by an inverted triangle bearing the number of its corresponding *B. thuringiensis* 407 Cry⁻ mutant.

in the osmolarity of the growth medium, to act as an osmoprotectant (Østerås *et al.*, 1998). Intracellular levels of glycine betaine are finely tuned to maintain the adequate cellular turgor essential for DNA replication and cell growth (Meury, 1988). It has been extensively demonstrated that a high osmolarity of the medium has an inhibitory effect on swarming motility (Liu *et al.*, 2000; Shin & Park, 1995; Young *et al.*, 1999). Therefore, the abnormal accumulation of glycine betaine in strain MP10 could mimic the physiological state that the cell acquires in high-osmolarity environments, thus impairing swarming motility.

BT2969, a gene putatively encoding catalase-2

The gene interrupted by the mini-Tn10 in strain MP11 encodes a protein showing sequence similarity (37% identity, 55% positivity) to *B. subtilis* KatE. In *B. subtilis* and *E. coli*, the *katE* gene encodes catalase-2 (hydroperoxidase II), which is not essential for active growth but is required for coping with cellular stress or adverse growth conditions, such as nutrient depletion or high cell density (Engelmann *et al.*, 1995; Von Ossowski *et al.*, 1991; Weber *et al.*, 2006). Although the role of catalase-2 in swarming differentiation by *B. thuringiensis* is not clear, we can suggest that this enzyme is needed when vegetative cells reach the high cell density required for swarming induction. This hypothesis is also supported by the finding that the *catB* gene of *Streptomyces coelicolor*, homologous to

katE, is essential for morphological differentiation of this organism on solid cultures (Cho *et al.*, 2000).

BT0713, a gene encoding a putative cryptic amino acid permease

The transposon TnQ962 inactivated a gene that encodes a hydrophobic integral membrane protein that may function as a cryptic amino acid permease. Since amino acids act as inducers of swarming differentiation in many microorganisms (Dick *et al.*, 1985; Köhler *et al.*, 2000) and the amino acid permease encoded by BT0713 is dispensable for vegetative growth in *B. thuringiensis* 407 Cry⁻, it can be supposed that such a cryptic permease is a possible sensory route for sensing extracellular signals that promote swarm cell differentiation by *B. thuringiensis*.

BT3576, a gene that encodes a putative ATP-binding cassette transporter component

Strain MP13 harbours the mini-Tn10 insertion in a BT3576 homologous gene. As noted following TMpred analysis, the amino acid sequence encoded by BT3576 contains a putative transmembrane helix at its N-terminus and a large C-terminal domain outside the cell. Analysis of the BT3576 predicted amino acid sequence revealed homology with the substrate-binding protein of a variety of ATP-binding cassette (ABC) transporters in many different organisms. Although further studies are required

to elucidate the role of BT3576 in swarming, the finding that the transposition Tn Ω 1851 produces a non-swarming phenotype suggests that the BT3576-encoded protein is part of a sensory system involved in the development of the highly organized *B. thuringiensis* swarming community.

BT5364, a gene predicted to encode a phosphohydrolase

The amino acid sequence deduced by BT5364, the gene disrupted in strain MP14, contains an HD sequence motif characteristic of metal-dependent phosphohydrolases. The family of HD phosphatases that have been analysed at the biochemical level are involved in a variety of biological processes, such as nucleic acid metabolism and signal transduction (Aravind & Koonin, 1998). Lack of additional conserved domains and the position of the HD domain inside the predicted protein encoded by BT5364 suggest its function as dGTPase. In *E. coli*, this enzyme is not essential for viability and is involved in purine breakdown under conditions of purine excess, such as those occurring transiently when cells approach the stationary phase (Kim *et al.*, 2006; Wurgler & Richardson, 1993). Whatever the role of the protein encoded by BT5364, it will be of interest to determine how abrogation of this function leads to lack of swarming development in *B. thuringiensis*.

MP15 shows defects in the production of phenazine compounds

Strain MP15 harbours the mini-Tn10 insertion in a gene that is homologous to the BT3423 gene of *B. thuringiensis konkukian* and potentially encodes an acetyltransferase (Table 4). As mentioned above, transposition Tn Ω 4245 could cause polar effects on the expression of the downstream gene BT3422 (Fig. 3). To assess this possibility, MP15 was transformed with pHT3423-3422 (Table 1), a plasmid carrying BT3422 and BT3423. The resulting strain (MP22) regained the ability to swarm, thus suggesting that the activity of both genes is required for swarming motility by *B. thuringiensis*. BT3422 is predicted to encode a phenazine biosynthesis protein of the *phzF* family. In *Pseudomonas* spp., the PhzF proteins are involved in the production of PZ derivative antibiotic and antifungal compounds (for a review see Chin-A-Woeng *et al.*, 2003). In *P. aeruginosa*, the production of the PZ compound pyocyanin is dependent on the AHL system (Chugani *et al.*, 2001), which also regulates swarming motility in this organism (Reimann *et al.*, 2002). Furthermore, the expression in *P. aeruginosa* of the *B. thuringiensis* gene *aiiA*, encoding an AHL-lactonase, leads to a decrease in pyocyanin synthesis, as well as in swarming motility (Wang *et al.*, 2007). More recently, an alteration in the ratio of PZs was associated with defects in the production of the surface multicellular communities constituting biofilms in *Pseudomonas chlororaphis* (Maddula *et al.*, 2008). To assess whether MP15 was

defective in the production of PZ compounds compared to the wild-type strain, total PZs were quantified in the supernatants of late-exponential-phase cultures of both strains. The mutant showed a 3.8-fold reduction in the amount of total PZs secreted, the A_{367} for 10^{10} cells being 0.85 ± 0.16 and 3.22 ± 0.34 for MP15 and *B. thuringiensis* 407 Cry⁻, respectively ($P < 0.001$). PZs were also quantified for both strains during growth under conditions that induced swarming in the wild-type strain. MP15 showed a reduction in the production of these compounds in such culture conditions also, the A_{367} for 10^{10} cells being 4.43 ± 0.91 vs 7.35 ± 1.76 ($P = 0.011$).

Although little is known yet regarding the physiological function of PZs in their natural environment, it is becoming clear that these compounds can act as secondary metabolites able to regulate global gene expression patterns (for a review see Price-Whelan *et al.*, 2006). The finding that an altered production of PZs is associated with the inability of *B. thuringiensis* to produce swarming colonies suggests that these molecules may serve as signals inducing gene expression changes required for differentiation into long and hyperflagellated swarm cells. It is expected that substantial additional work will be required to elucidate the biosynthetic pathways of PZs in *B. thuringiensis* and the exact mechanism by which PZs regulate the development of *B. thuringiensis* multicellular communities.

Conclusions

We have identified six previously uncharacterized *B. thuringiensis* genes that are required for swarming, but dispensable for flagellation, swimming, chemotaxis and active growth. The finding that these proteins appear to be involved in several and diverse cellular functions highlights the complex scenario of molecular components governing swarming by *B. thuringiensis*. Further studies are required to better elucidate the role of the identified genes in allowing swimmer cells to enter a swarm-cell state.

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