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

Sara Salvetti,1 Francesco Celandroni,1 Mara Ceragioli,2 Sonia Senesi2 and Emilia Ghelardi1

**Correspondence**
Emilia Ghelardi
ghelardi@biomed.unipi.it

1Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, Università di Pisa, via San Zeno 35-39, 56127 Pisa, Italy
2Dipartimento di Biologia, Sezione di Microbiologia, Università di Pisa, via San Zeno 35-39, 56127 Pisa, Italy

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**INTRODUCTION**

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*.

**Abbreviations:** AHL, *N*-acylhomoserine lactone; *T*\(_{\text{gen}}\) generation time; CI, chemotaxis index; PZ, phenazine.
swarm-cell differentiation on the quorum sensing mediated by extracellular N-acylhomoserine lactones (AHLLs) 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), heart infusion broth (HI) 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: 

\[
T_{\text{gen}} = \frac{t}{3.3 \log (b/b)}
\]

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-CBH 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: 

\[
CI = \frac{D_L - D_S}{D_L},
\]

where 

\( D_L \) is the mean of growth halo diameters recorded on TrM plates supplemented with mannitol and 

\( D_S \) 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 × 10⁸ cells ml⁻¹) and incubated at 37 °C in a humidified chamber. The swimming 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 (Harvey & 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 EcoRI or HindIII (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 PredictProtein at the ExPaSy-proteomics server of the Swiss Institute of Bioinformatics (http://expasy.org).
which liberated 1
One unit (U) of the enzyme was defined as the amount of enzyme
substrate. Each reaction mixture was incubated at 37
\( \text{N,N} \) sarcosine or 0.2 M

essentially as described by Maddula
et al.

were grown in 5 ml BHIG broth at 30
\( \text{C} \) to the late-exponential

phase and cell-free culture supernatants were acidified to pH 2 with

\( 0.1 \text{ M NaOH} \), quantified via absorbance at 367 nm and expressed

in crude extracts of bacterial cultures that were normalized to the


cell density (OD\text{600}).

Sarcosine oxidase activity. Sarcosine oxidase activity was assayed

in crude extracts of bacterial cultures that were normalized to the

same OD\text{600}, as described by Koyama et al. (1991), using 0.2 M

sarcosine or 0.2 M \text{N,N}-dimethylglycine (Sigma Aldrich) as the

substrate. Each reaction mixture was incubated at 37 \( ^\circ \text{C} \) for 10 min.

One unit (U) of the enzyme was defined as the amount of enzyme

which liberated 1 \text{ \mu mol 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 \( ^\circ \text{C} \) 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 \( A_{\text{abs}} \) for \( 10^{10} \) bacterial cells. PZs were also quantified after growth

under swarming condition by growing bacteria on Anopore membranes (0.2 \text{ \mu m 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 \( ^\circ \text{C} \), 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 ± SD from five independent experiments.

RESULTS AND DISCUSSION

Phenotype of \textit{B. thuringiensis} 407 Cry\textsuperscript{−} swarming colonies on different solid media

Swarming in \textit{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,
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). 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. Swarming-proficient and normally growing 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 (Tgen ranging from 22.06±2.4 to 27.91±2.8 min) and biochemical profile;

### Table 2. Primers used for PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Target/base pair coordinates</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT2787U</td>
<td>GATAAGCTTTAATACGCTT</td>
<td>BT2787, from −145 to −125</td>
<td>HindIII</td>
</tr>
<tr>
<td>BT2787L</td>
<td>TCCGGATCTTATACGAC</td>
<td>BT2787, from +1364 to +1344</td>
<td>BamHI</td>
</tr>
<tr>
<td>BT2969U</td>
<td>CTAAGCTTTATACGCTT</td>
<td>BT2969, from −102 to −84</td>
<td>Sphi</td>
</tr>
<tr>
<td>BT2969L</td>
<td>ATGTCGAACTGTTGCAAT</td>
<td>BT2969, from +1570 to +1552</td>
<td>BamHI</td>
</tr>
<tr>
<td>BT2787U</td>
<td>GATGCTTATACGCTT</td>
<td>BT2787, from −71 to −53</td>
<td>PstI</td>
</tr>
<tr>
<td>BT2787L</td>
<td>ATGTCGAACTGTTGCAAT</td>
<td>BT2787, from +1731 to +1713</td>
<td>BamHI</td>
</tr>
<tr>
<td>BT0713U</td>
<td>CCAAGCTTATACGCTT</td>
<td>BT0713, from −141 to −125</td>
<td>HindIII</td>
</tr>
<tr>
<td>BT0713L</td>
<td>GATGCTTATACGCTT</td>
<td>BT0713, from +1447 to +1429</td>
<td>PstI</td>
</tr>
<tr>
<td>BT3564U</td>
<td>AGCAAGCTTACTCTTTT</td>
<td>BT3564, from −103 to −85</td>
<td>HindIII</td>
</tr>
<tr>
<td>BT5364L</td>
<td>TCAAGCTTACGCTT</td>
<td>BT3423, from +965 to +947</td>
<td>BamHI</td>
</tr>
<tr>
<td>BT5364L</td>
<td>TAAAGCTTACGCTT</td>
<td>BT3423, from +984 to +966</td>
<td>BamHI</td>
</tr>
<tr>
<td>BT3423U</td>
<td>TGAAAGCTTCTGAGGAG</td>
<td>BT3423, from −140 to −125</td>
<td>HindIII</td>
</tr>
<tr>
<td>BT3423L</td>
<td>TATGGATCTTCTTGC</td>
<td>BT3423, from +1436 to +1416</td>
<td>HindIII</td>
</tr>
<tr>
<td>BT3422U</td>
<td>TATGGATCTTCTTGC</td>
<td>BT3422, from +1450 to +1432</td>
<td>HindIII</td>
</tr>
<tr>
<td>BT3422L</td>
<td>TATGGATCTTCTTGC</td>
<td>BT3422, from +1450 to +1438</td>
<td>HindIII</td>
</tr>
<tr>
<td>E1</td>
<td>GCTGAGCTAGTTCATTAAT</td>
<td>Mini-Tn10</td>
<td>None</td>
</tr>
<tr>
<td>E3</td>
<td>CTGATTTCCAGGTTTACCCAC</td>
<td>Mini-Tn10</td>
<td>None</td>
</tr>
</tbody>
</table>

*Restriction site is underlined.
†Numbers refer to nucleotide position in the indicated gene, relative to the putative translational initiation site.
(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 HindIII or EcoRI 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 HindIII or EcoRI 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 Cry2 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**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Liquid environment</th>
<th>Solid surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOT*</td>
<td>CI†</td>
</tr>
<tr>
<td>407 Cry⁻</td>
<td>+</td>
<td>0.94</td>
</tr>
<tr>
<td>MP10</td>
<td>+</td>
<td>0.78</td>
</tr>
<tr>
<td>MP11</td>
<td>+</td>
<td>0.66</td>
</tr>
<tr>
<td>MP12</td>
<td>+</td>
<td>0.85</td>
</tr>
<tr>
<td>MP13</td>
<td>+</td>
<td>1.20</td>
</tr>
<tr>
<td>MP14</td>
<td>+</td>
<td>1.40</td>
</tr>
<tr>
<td>MP15</td>
<td>+</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*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. 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.
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 Tn\(^V\)\(_{4245}\) could generate polar effects on the expression of the downstream gene (Table 4, Fig. 3).

**BT2787 encodes monomeric sarcosine oxidase**

The transposition Tn\(\Omega\)\(_{63}\) 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-Tn\(10\) 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\(^2\) (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

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**Table 4. Transposon insertion sites that abolish swarming differentiation**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transposon insertion</th>
<th>Transposon insertion site</th>
<th>Polar effect of transposon insertion*</th>
<th>Homologous gene†</th>
<th>Identity (%)‡</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP10</td>
<td>Tn(\Omega)63</td>
<td>GGTTGCGCT</td>
<td>No</td>
<td>BT2787</td>
<td>86</td>
<td>Sarcosine oxidase</td>
</tr>
<tr>
<td>MP11</td>
<td>Tn(\Omega)940</td>
<td>TACGAAGTT</td>
<td>No</td>
<td>BT2969</td>
<td>87</td>
<td>Catalase-2</td>
</tr>
<tr>
<td>MP12</td>
<td>Tn(\Omega)962</td>
<td>CATTAAGCT</td>
<td>No</td>
<td>BT0713</td>
<td>83</td>
<td>Amino acid permease</td>
</tr>
<tr>
<td>MP13</td>
<td>Tn(\Omega)1851</td>
<td>TGCTGAGTA</td>
<td>No</td>
<td>BT3576</td>
<td>86</td>
<td>ABC transporter, substrate-binding protein</td>
</tr>
<tr>
<td>MP14</td>
<td>Tn(\Omega)4040</td>
<td>GATCAAGTA</td>
<td>No</td>
<td>BT3564</td>
<td>90</td>
<td>dGTP triphosphohydrolase</td>
</tr>
<tr>
<td>MP15</td>
<td>Tn(\Omega)4245</td>
<td>TACTTGGAT</td>
<td>Yes</td>
<td>BT3423</td>
<td>90</td>
<td>Acetyltransferase</td>
</tr>
</tbody>
</table>

* 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.
in the osmolarity of the growth medium, to act as an osmoprotectant (Østera˚s 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 transposition Tn962 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 TnΩ1851 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 TnΩ4245 could cause polar effects on the expression of the downstream gene BT3422 (Fig. 3). To assess this possibility, MP15 was transformed with pH3423-3422 (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 (Reimmann 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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