

# c-di-GMP induction of *Dictyostelium* cell death requires the polyketide DIF-1

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**ABSTRACT** Cell death in the model organism *Dictyostelium*, as studied in monolayers in vitro, can be induced by the polyketide DIF-1 or by the cyclical dinucleotide c-di-GMP. c-di-GMP, a universal bacterial second messenger, can trigger innate immunity in bacterially infected animal cells and is involved in developmental cell death in *Dictyostelium*. We show here that c-di-GMP was not sufficient to induce cell death in *Dictyostelium* cell monolayers. Unexpectedly, it also required the DIF-1 polyketide. The latter could be exogenous, as revealed by a telling synergy between c-di-GMP and DIF-1. The required DIF-1 polyketide could also be endogenous, as shown by the inability of c-di-GMP to induce cell death in *Dictyostelium* HMX44A cells and DH1 cells upon pharmacological or genetic inhibition of DIF-1 biosynthesis. In these cases, c-di-GMP-induced cell death was rescued by complementation with exogenous DIF-1. Taken together, these results demonstrated that c-di-GMP could trigger cell death in *Dictyostelium* only in the presence of the DIF-1 polyketide or its metabolites. This identified another element of control to this cell death and perhaps also to c-di-GMP effects in other situations and organisms.

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## INTRODUCTION

A search for phylogenetically conserved elements in cell death mechanisms can benefit from the advantages of the model protist *Dictyostelium discoideum*. *Dictyostelium* belongs to a eukaryote supergroup distinct from but phylogenetically close to that comprising animals. *Dictyostelium* cells multiply vegetatively in rich medium and aggregate upon starvation. Within 24 h, this aggregate morphogenizes into a 1- to 2-mm-high fruiting body, which includes a stalk made of vacuolized, cellulose-walled dead cells. This *Dictyostelium* developmental cell death can be mimicked in vitro in monolayer conditions (Kay, 1987; Cornillon et al., 1994; Giusti et al., 2009). This death is neither apoptotic nor necrotic but vacuolar. Its induction requires two signals. The first signal, starvation, sensitizes the cells and also triggers autophagy. Autophagy protects cells from damage due to starvation and thus allows second

signal-induced cell death (Luciani et al., 2011). Only cells that have undergone starvation can be induced to die by a second signal, classically the polyketide DIF-1 (Kay, 1987).

Recently not only DIF-1, but also the cyclic dinucleotide c-di-GMP was shown to induce cell death in *Dictyostelium* cell monolayers (Chen and Schaap, 2012) and to be required for the development of *Dictyostelium* (Chen and Schaap, 2012). c-di-GMP, a universal bacterial second messenger (Romling et al., 2013), has recently been shown to affect also animal cells, where it can trigger innate immunity upon bacterial infection (Danilchanka and Mekalanos, 2013) and regulate  $\beta$ -adrenergic stimulation (Lolicato et al., 2014). Studying c-di-GMP-induced *Dictyostelium* cell death may provide both an additional handle on mechanisms at play in this cell death and also more general information on how c-di-GMP acts in a eukaryotic cell. We unexpectedly found that c-di-GMP was not sufficient by itself to induce cell death in *Dictyostelium* cell monolayers. This induction of cell death by c-di-GMP required the synthesis of the polyketide DIF-1 or its metabolites.

## RESULTS

### Exogenous DIF-1 and c-di-GMP trigger distinct pathways to cell death

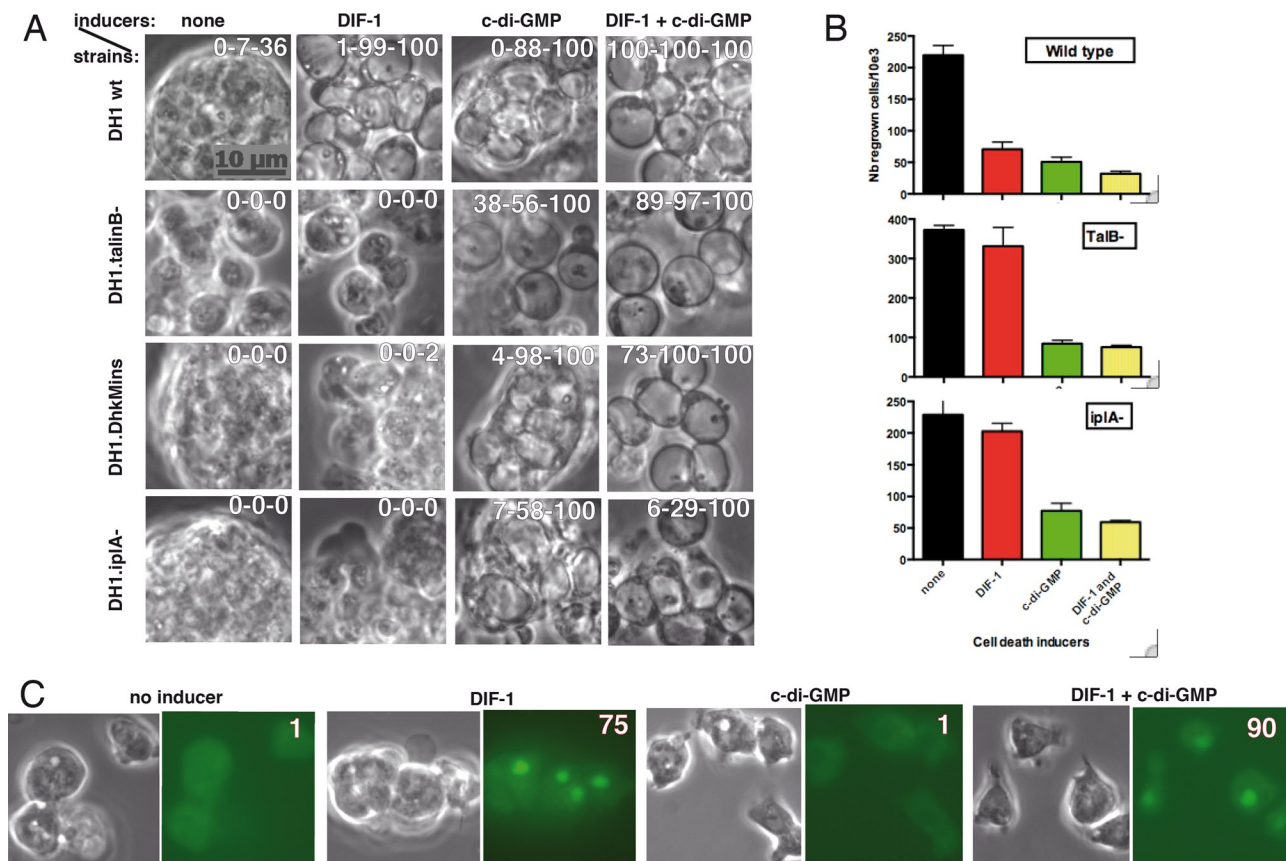
Induction in vitro by DIF-1 or by c-di-GMP led to cell death with similar subcellular lesions, such as vacuolization and synthesis of

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Abbreviations used: c-di-GMP, cyclic diguanylate, cyclic-di-GMP; nt, nucleotide; SB, Sørensen buffer.

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**FIGURE 1:** DIF-1-induced and c-di-GMP-induced pathways to cell death showed distinct features. (A) Starved DH1 wild-type cells showed vacuolization upon addition of 100 nM DIF-1 or 10  $\mu$ M c-di-GMP. In contrast, DH1 talinB<sup>-</sup>, DhkMins, or iplA<sup>-</sup> mutant cells showed vacuolization upon addition of c-di-GMP but not of DIF-1. The vignettes show cells after 40 h in the presence or absence of inducers. The number at the upper right corner of each vignette shows the percentage of clumps, each comprising at least three vacuolized cells, determined at 16, 24, and 40 h, respectively, after addition of DIF-1 or c-di-GMP. Each of these percentage values was obtained by screening at least 100 clumps. For instance, at 16 h post-DIF-1 or post-c-di-GMP, there were no or only few clumps each with at least three vacuolized cells, whereas in the presence of both inducers together, every clump showed at least three vacuolized cells. For clarity, each individual percentage value is not accompanied by its confidence interval. Out of 100 clumps, for a measured percentage of 50%, the 95% confidence interval of the percentage would be  $\pm 9.8\%$ , and this confidence interval would decrease for measured percentages deviating from 50%. Thus the 95% confidence interval of each of the measured percentages shown is  $\leq \pm 9.8\%$ . (B) Cells in LabTek chambers were starved and incubated for 24 h in the absence of inducer or in the presence of 100 nM DIF-1, 10  $\mu$ M c-di-GMP, or both. HL5 medium was then added and incubation proceeded for 3 d, and the regrown cells were counted. Each column shows the mean of number of cells regrown in three cultures and the corresponding 95% confidence interval. Wild-type cells showed low regrowth with either inducer, whereas talinB<sup>-</sup> and iplA<sup>-</sup> mutant cells showed low regrowth with c-di-GMP but no significant difference with DIF-1 compared with control without inducer. Addition of both inducers further moderately reduced regrowth. (C) DimB nuclear translocation occurred upon addition of DIF-1 but not of c-di-GMP. DH1 cells transfected with GFP-DimB were subjected to starvation and 100 nM DIF-1 and/or 10  $\mu$ M c-di-GMP for 10 min. The same fields were taken by phase contrast and fluorescence microscopy. From these pictures, the percentage of cells showing nuclear translocation of fluorescence was determined and is shown as numbers in each of the vignettes. Each of these percentage values was obtained by screening at least 100 cells, yielding 95% confidence intervals  $\leq \pm 9.8\%$ .

cellulose cell encasings (Levraud *et al.*, 2003; Giusti *et al.*, 2009; unpublished data). However, in *Dictyostelium* cells of the DH1 strain, whereas vacuolization induced by DIF-1 was prevented by the talB (Giusti *et al.*, 2009), iplA (Lam *et al.*, 2008), and DhkMins (Giusti *et al.*, 2010) mutations (Figure 1A, second column), vacuolization induced by exogenous c-di-GMP was not prevented by these mutations (Figure 1A, third column). Consistently, these mutations prevented cell death (defined here as the inability of cells to regrow upon addition of rich medium) induced by DIF-1 but not that induced by exogenous c-di-GMP (Figure 1B; unpublished data). In

this article, exogenous means experimentally added to the cells under test, and endogenous means produced by (some of) the cells under test and acting on the same or other cells. Similarly, cyclosporin A inhibited vacuolization and cell death induced by DIF-1 (Lam *et al.*, 2008) but not by c-di-GMP (Supplemental Figure S1). In addition, as in other *Dictyostelium* strains (Huang *et al.*, 2006; Zhukovskaya *et al.*, 2006), in DH1 cells, the bZip transcription factor DimB translocated from the cytosol to the nucleus in a matter of minutes after addition of DIF-1 (with or without c-di-GMP; Figure 1C) but not after addition of c-di-GMP (Figure 1C). Taken together,

these differences indicated that at least parts of the pathways to cell death induced by exogenous DIF-1 and exogenous c-di-GMP were distinct.

### Exogenous DIF-1 and c-di-GMP synergize to induce cell death

Unexpectedly, cells subjected to both exogenous DIF-1 and exogenous c-di-GMP together showed markedly more (Figure 1A, top, and Supplemental Figure S2A) and earlier (Supplemental Figure S3) vacuolization than cells subjected to either alone. Further, the synergy between DIF-1 and c-di-GMP occurred not only in parental DH1 cells, but also in the *talinB* and *DhkMins* mutant cells that did not vacuolize and did not die in the presence of DIF-1 alone (Figure 1A). Thus mutations interrupting the pathway used by DIF-1 alone (hereafter called the autonomous DIF-1 pathway) did not interrupt the DIF-1 pathway to synergistic vacuolization, indicating that these pathways were distinct.

Further, in *iplA* mutant cells, there was no more vacuolization upon addition of both DIF-1 and c-di-GMP than upon addition of c-di-GMP only (Figure 1A). Thus the IP3R was required, not only for the autonomous DIF-1 pathway to cell death (Lam *et al.*, 2008), but also for the synergistic DIF-1 plus c-di-GMP-induced cell death, suggesting that the positive interaction between exogenous DIF-1 and c-di-GMP-induced pathways included an IP3R-dependent step.

When cell death induced by DIF-1 and c-di-GMP at low concentrations was tested by regrowth, synergy seemed much less than when tested by vacuolization (Figure 1B and Supplemental Figure S2B). Vacuolization was checked usually at 22–24 h postinduction, whereas “regrowth,” namely the number of cells present, was checked after a further 3 d of incubation in HL5. In these experiments, vacuolization leading to fewer regrowing cells could proceed even after addition of HL5 (unpublished data). Thus the delay in vacuolization observed at 22 h for single compared with double induction could be compensated during the 3 d required for a regrowth test. In other words, single-induced vacuolization could catch up with double-induced vacuolization during the 22-h to 3-d incubation in HL5, leading to a lesser difference between single and double induction in terms of regrown cells at 3 d. As a corollary, vacuolization is a sign of cell death kinetically more accurate than non-regrowth. It will be often taken hereafter as equivalent to cell death, confirmed or not by regrowth tests.

Taking the results together, synergy increased the speed rather than the final extent of vacuolization and death. Most important, it indicated a positive interaction between pathways to cell death induced by exogenous DIF-1 and by c-di-GMP. Could this interaction between the effects of exogenous inducers reflect a required endogenous cooperation?

### HMX44A cells that produce little or no DIF-1 do not vacuolize or die upon addition of c-di-GMP

We first used *Dictyostelium* HMX44A cells, which are known to make very little DIF-1 but to remain responsive to exogenous DIF-1 (Kopachik *et al.*, 1983). Accordingly, these cells vacuolized well with exogenous DIF-1 (Figure 2A). In contrast, addition of exogenous c-di-GMP led to small, roundish, contrasted cells with almost no vacuolization (Figure 2A), no cellulose encasings (unpublished data), and no death when tested by regrowth (Figure 2B, right). In addition, in these HMX44A cells, where exogenous c-di-GMP alone did not induce cell death, there was synergy between exogenous DIF-1 and exogenous c-di-GMP (Figure 2C). Taken together, the results indicate that in HMX44A cells relative to DH1 cells, an unknown

mutation, or mutations, prevented vacuolar cell death induced by c-di-GMP. Again, irrespective of their other possible defects, HMX44A cells produced no or little DIF-1. These results were in line with the possibility of a requirement of DIF-1 for c-di-GMP induction of cell death.

### Exogenous c-di-GMP requires polyketides to induce cell death

To check in DH1 cells (for consistency with the aforementioned mutants) whether DIF-1 or other polyketides were required for c-di-GMP-induced cell death, we first used cerulenin, known to inhibit the biosynthesis of polyketides, including that of DIF-1 (Kay, 1998; Serafimidis and Kay, 2005). Cerulenin, as expected, did not impair vacuolization induced by exogenous DIF-1 (and thus did not impair vacuolization as such), but, remarkably, almost completely prevented induction of vacuolization by exogenous c-di-GMP (Figure 3A). This indicated that one or several cerulenin-inhibitable moieties were required together with c-di-GMP for induction of cell death. Cerulenin inhibits the  $\beta$ -keto-acyl domain of polyketide synthases, including in *Dictyostelium* not only the *stlB* polyketide synthase (Austin *et al.*, 2006; Saito *et al.*, 2008) required for the biosynthesis of the THPH precursor of DIF-1, but also the *StIA* polyketide synthase, plus close to 40 other polyketide synthases, including two fatty acid synthases (Chance *et al.*, 1976; Omura, 1976; Kridel *et al.*, 2007; Zucko *et al.*, 2007; Narita *et al.*, 2014). What is the polyketide synthase catalyzing the synthesis of molecules required for c-di-GMP-induced cell death?

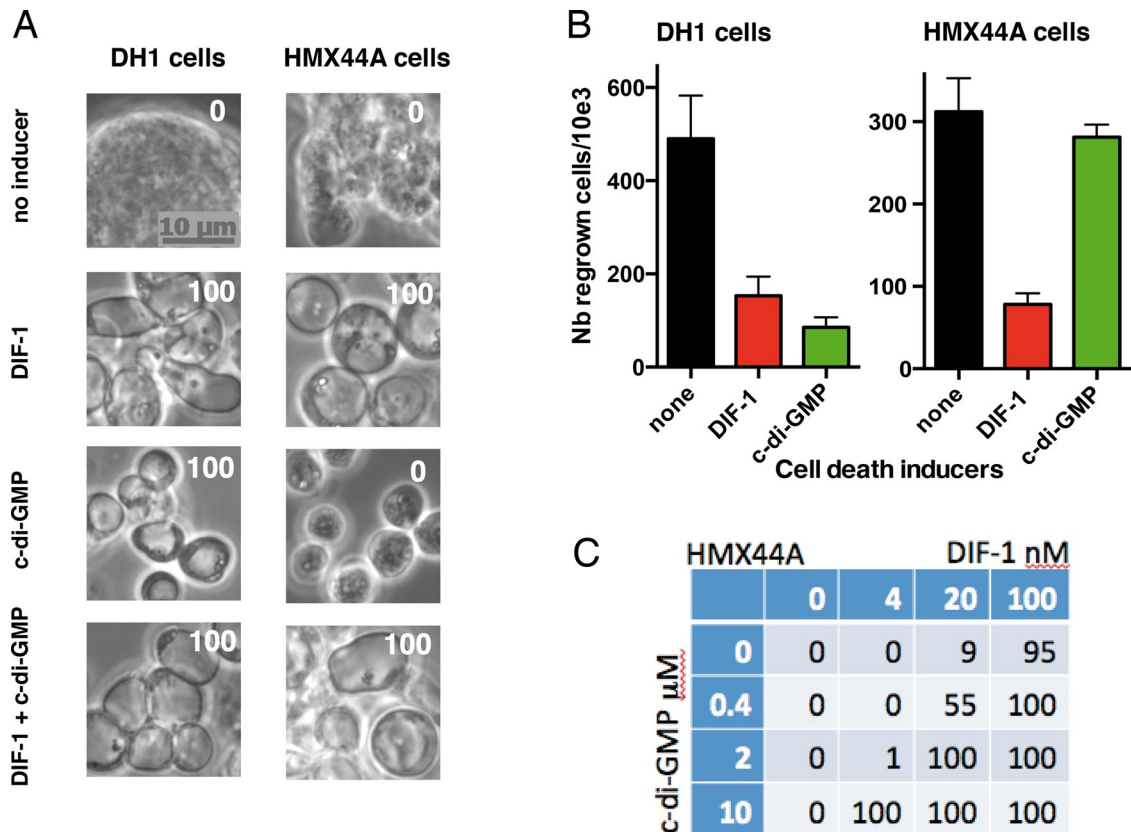
### Exogenous c-di-GMP requires endogenous *stlB*-dependent polyketides to induce cell death

The polyketide synthase *stlB* catalyzes the first step of DIF-1 biosynthesis (Saito *et al.*, 2008; Supplemental Figure S4). We disrupted in DH1 cells the gene encoding *stlB* (Supplemental Figure S5), which was previously disrupted in strain AX2 (Saito *et al.*, 2008). This *stlB* mutation almost completely prevented c-di-GMP-induced vacuolization (Figure 3B) and cell death (unpublished data), thus accounting at least in part for the cerulenin results and showing that the *stlB*-initiated biosynthetic cascade included at least one polyketide required for the major part of cell death induction by c-di-GMP.

### DIF-1 is the main endogenous *stlB*-dependent polyketide cooperating with c-di-GMP for induction of cell death

Which *stlB*-dependent polyketide(s) cooperate(s) with c-di-GMP? Within the *stlB*-dependent biosynthetic cascade (Supplemental Figure S4), the *DmtA* methyltransferase catalyzed the last step to DIF-1 biosynthesis. We disrupted the *DmtA* gene in DH1 cells (Supplemental Figure S6), previously disrupted in strain AX2 (Thompson and Kay, 2000). DH1.*DmtA*<sup>-</sup> cells, which vacuolized as well as DH1 cells upon addition of DIF-1 or of both DIF-1 and c-di-GMP (Figure 4), when subjected to c-di-GMP vacuolized much less than DH1 cells (Figure 4). Thus cell death induction by c-di-GMP required polyketides depending on the *DmtA* methylase, namely DIF-1 and/or its metabolites (Traynor and Kay, 1991).

Induction of cell death by exogenous c-di-GMP required endogenous DIF-1. Did cell death induction by exogenous DIF-1 symmetrically require endogenous c-di-GMP? In DH1 cells, we mutated the diguanylate cyclase *DgcA* gene synthesizing c-di-GMP (Chen and Schaap, 2012). As previously reported for NC4A2.*DgcA*<sup>-</sup> cells (Chen and Schaap, 2012), the development of DH1.*DgcA*<sup>-</sup> cells was interrupted at the slug stage (Supplemental Figure S7A). Under monolayer conditions, upon addition of DIF-1, these



**FIGURE 2:** In contrast to DH1 cells, *Dictyostelium* HMX44A cells could be induced to die by DIF-1 but not by c-di-GMP. In separate experiments, DH1- or HMX44A-starved cells were subjected to either 100 nM DIF-1 or 10  $\mu\text{M}$  c-di-GMP (A) for 40 h and then photographed (see Figure 1A legend for details) or (B) for 24 h and then subjected to HL5 medium to allow regrowth of surviving cells; regrown cells were counted after 3 more days (see Figure 1B legend for details). (C) HMX44A cells were subjected in LabTek chambers to graded concentrations of DIF-1 and c-di-GMP in a checkerboard manner. The numbers are the percentages of clumps each comprising at least three vacuolized cells after 17 h in the presence or absence of inducers, established as in the legend to Figure 1A. HMX44A cells showed vacuolization when subjected to DIF-1 alone but not to c-di-GMP alone, and there was synergy between the two inducers. These cells made no endogenous DIF-1, leading to no vacuolization by exogenous c-di-GMP. However, addition of exogenous DIF-1 led to vacuolization through the autonomous DIF-1 pathway and through cooperation with exogenous c-di-GMP (see Figure 5 for schematization).

DH1.DgcA<sup>-</sup> cells vacuolized as well as the DH1 parental cells (Supplemental Figure S7B), although they were unable to synthesize c-di-GMP. Thus, whereas exogenous c-di-GMP required endogenous DIF-1 to induce vacuolization, the reverse was not true.

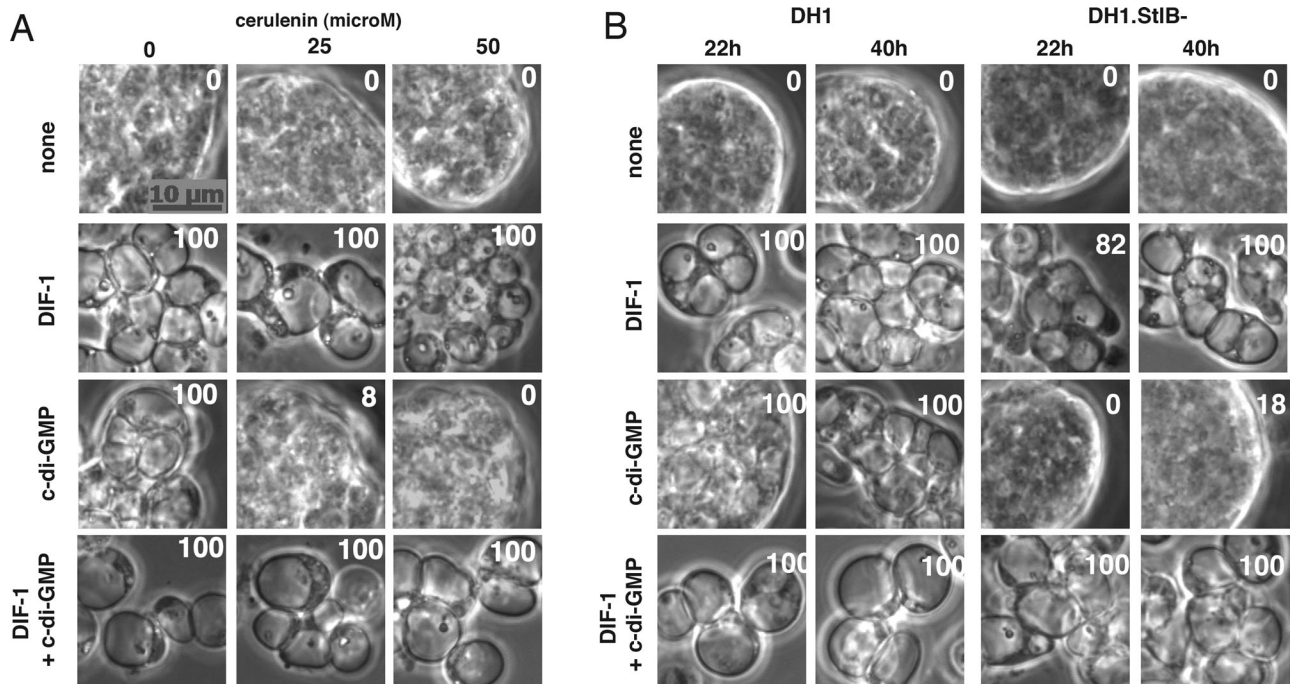
Some of these results and conclusions were recapitulated in the following experiment (Figure 5A). TalinB<sup>-</sup> mutant cells were subjected to DIF-1, c-di-GMP, or both in the presence or absence of cerulenin. DIF-1 alone induced no vacuolization, since the autonomous DIF-1 pathway was blocked by the talinB mutation. c-di-GMP alone induced vacuolization in the absence, but not in the presence, of cerulenin, since the latter prevented endogenous DIF-1 synthesis, which was required for c-di-GMP-induced vacuolization. Thus, in these talinB<sup>-</sup> cells in the presence of cerulenin, neither DIF-1 nor c-di-GMP alone could induce vacuolization. However, DIF-1 and c-di-GMP together induced vacuolization due to complementation by exogenous DIF-1 of the block by cerulenin of endogenous DIF-1 synthesis, thus providing DIF-1 to ensure c-di-GMP-induced vacuolization. Taken together, our results and conclusions lead to a tentative schematic representation of DIF-1 and c-di-GMP pathways to *Dictyostelium* vacuolar cell death in monolayers (Figure 5B).

## DISCUSSION

Following the reports that the polyketide DIF-1 (Morris *et al.*, 1987) or the cyclic-dinucleotide c-di-GMP (Chen and Schaap, 2012) induced *Dictyostelium* cell death *in vitro*, we demonstrated here that induction of cell death *in vitro* by c-di-GMP requires DIF-1, as schematized in Figure 5B. We first unexpectedly observed that exogenous DIF-1 and c-di-GMP acted in synergy, suggesting in particular that endogenous amounts were limiting. Through mutations of the autonomous DIF-1 pathway, we then disentangled a cooperative from an autonomous DIF-1 pathway. We then showed through the use of cerulenin that c-di-GMP induction of cell death requires the synthesis of polyketides. Through the use of stlB mutants, we showed that at least one of these polyketides was made in a stlB-dependent biosynthetic cascade and, through the use of DmtA mutants, that this polyketide was DIF-1 and/or its metabolites.

Together, present and previous results suggested the existence of several partially distinct DIF-1 pathways. An exogenous DIF-1-induced pathway autonomously led to vacuolar cell death and could be inhibited by the talinB, iplA, and DhkM mutations (Lam *et al.*, 2008; Giusti *et al.*, 2009, 2010). Another pathway required for c-di-GMP-induced cell death was the endogenous biosynthesis of DIF-1





**FIGURE 3:** Inhibition of the endogenous DIF-1 biosynthetic pathway prevented vacuolization by exogenous c-di-GMP. (A) Pharmacological inhibition of polyketide synthesis by cerulenin added to DH1 cells at the indicated concentration as soon as the beginning of starvation and for the whole duration of the experiment. Cells were examined 40 h after addition of DIF-1 and/or c-di-GMP. (B) In a separate experiment, DH1 parental and DH1.stlB<sup>-</sup> mutant cells were examined 22 and 40 h after addition of DIF-1 and/or c-di-GMP. Numbers are percentages of vacuolization as in the legend to Figure 1A. Cerulenin or the stlB mutation prevented c-di-GMP-induced vacuolization, showing that it required an stlB-dependent polyketide. Similar results were obtained with another independently obtained stlB<sup>-</sup> mutant clone.

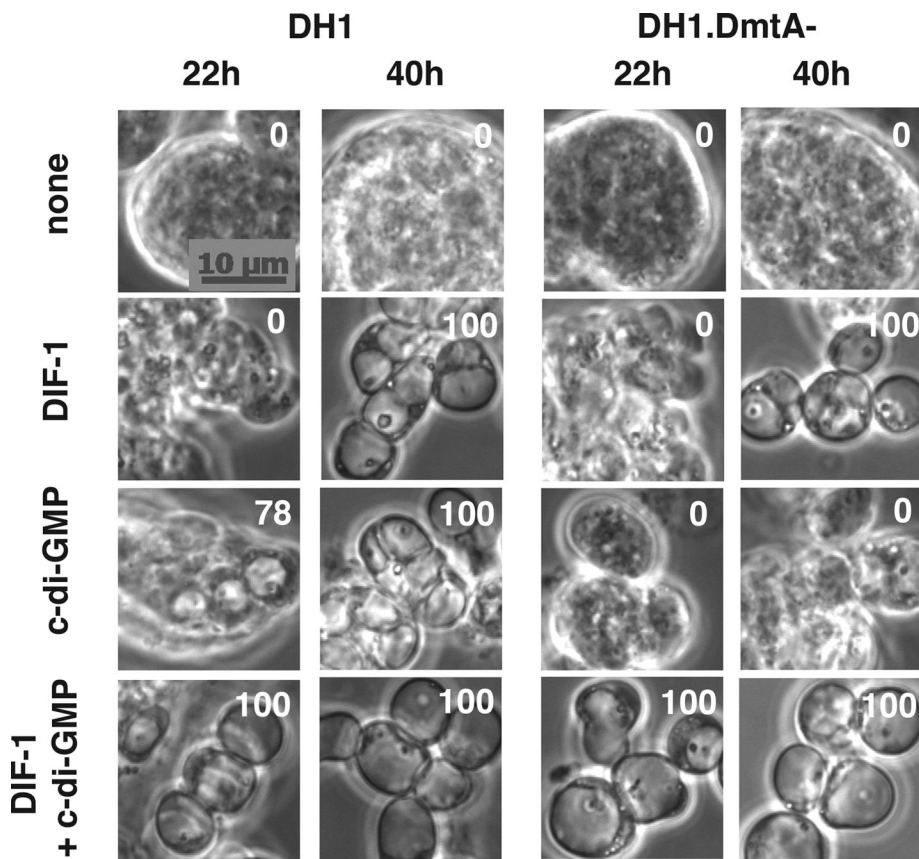
and its metabolites. This biosynthesis required the expression and activation of, in particular, the StlB and DmtA enzymes. Perhaps through cAMP-requiring activation of the transcription factor GbfA (Schnitzler *et al.*, 1995; Gollop and Kimmel, 1997; Giusti *et al.*, 2009), expression and activation of DmtA were induced by cAMP (Kay and Thompson, 2001), catalyzing the synthesis of DIF-1 and thus “sensitizing” the cells to subsequently added exogenous c-di-GMP. Under our experimental conditions, c-di-GMP induction of cell death required preincubation with cAMP (unpublished data). Yet another DIF-1-induced pathway, not sensitive to any of the mutations listed here, led to paddle cells (Levraud *et al.*, 2003; Giusti *et al.*, 2009), and a fourth pathway could lead to atg1<sup>-</sup> mutant cells to necrotic cell death (Kosta *et al.*, 2004) due to particular DIF-1 functional groups (Luciani *et al.*, 2009).

In contrast, but still *in vitro*, c-di-GMP induced no detectable autonomous pathway to vacuolar cell death. Exogenous c-di-GMP induced cell death only when DIF-1 was exogenously or endogenously available (Figure 5). No mutation is known to inhibit this c-di-GMP-induced pathway, except those impairing DIF-1 synthesis. Of note, endogenously synthesized DIF-1, which is required and is in sufficient amount for c-di-GMP-induced cell death, is not sufficient to induce vacuolar or necrotic cell death autonomously, suggesting that quantitative and/or perhaps topological parameters are at play. Also in contrast to DIF-1, c-di-GMP did not induce necrotic cell death in atg1<sup>-</sup> cells (unpublished data).

These results have implications for requirements for cell death *in vivo*, which is part of stalk cell differentiation within *Dictyostelium* multicellular development. Two candidate moieties could mediate induction of this cell death *in vivo*, namely the polyketide DIF-1 (Kay, 1987) and the cyclic dinucleotide c-di-GMP (Chen and Schaap,

2012). If DIF-1 were required, then DIF-1 biosynthesis mutants should not allow cell death induction *in vivo*—but they do (Thompson and Kay, 2000; Saito *et al.*, 2008), except for basal disk cells. Thus DIF-1 cannot be the only cell death inducer *in vivo*. Is c-di-GMP this main cell death inducer? An argument in favor of this is the developmental phenotype of DgcA-null mutants (Chen and Schaap, 2012). These mutants, incapable of c-di-GMP biosynthesis, did not develop past the slug stage. They showed normal formation of slugs that could not form fruiting bodies but continued migration (Chen and Schaap, 2012). Moreover, exogenous c-di-GMP restored fruiting body formation (Chen and Schaap, 2012). This is consistent with a role of c-di-GMP for stalk cell formation but may also be interpreted as evidence for another role of c-di-GMP between slug migration and initiation of fruiting body formation. Experiments testing the expression of stalk-specific molecules in wild type, DgcA-null mutants, and DgcA-null mutants plus exogenous c-di-GMP might provide evidence either way.

An argument that at first sight is not in favor of a role for c-di-GMP in induction of cell death *in vivo* is the required cooperation with DIF-1 for c-di-GMP induction of cell death *in vitro*, as shown here. If c-di-GMP is the main cell death inducer *in vivo*, and if this requires DIF-1 cooperation, then mutants impairing DIF-1 biosynthesis should prevent most of cell death *in vivo*, but, again, they do not (Thompson and Kay, 2000; Saito *et al.*, 2008). How is one to account for the persistence of cell death induction *in vivo* when there is no DIF-1 to cooperate with c-di-GMP, in contrast with the absence of cell death induction *in vitro* in the same circumstances? DIF-1 cooperation may be redundant *in vivo*. For instance, considering the considerable number of polyketide synthases encoded by the *Dictyostelium* genome (Eichinger *et al.*, 2005;



**FIGURE 4:** Mutation of the DmtA methylase prevented vacuolization by exogenous c-di-GMP. c-di-GMP-induced vacuolization was prevented by a DmtA mutation, which did not affect vacuolization induced by exogenous DIF-1 or by DIF-1 and c-di-GMP. Numbers are percentages of vacuolization as in the legend to Figure 1A. These results showed that DmtA-dependent polyketides, namely DIF-1 and/or its metabolites, were required for c-di-GMP-induced cell death. The differences in the extent of inhibition of vacuolization between DmtA<sup>-</sup> cells in this figure and StlB<sup>-</sup> cells in Figure 3 likely reflect the differences in the kinetics/extent of vacuolization of control DH1 parental cells between these experiments.

Zucko *et al.*, 2007), some of the corresponding non-stlB-dependent polyketides might cooperate with c-di-GMP in vivo at various stages and sites of development, such as the polyketide MPDB, which, of interest, is the product of the stlA polyketide synthase (Anjard *et al.*, 2011). More generally, compared with cells in vitro, cells in slugs may be exposed to different cell contacts, be at a different developmental stages, or be subjected to other signals. This may allow a major role of c-di-GMP for cell death induction in vivo even in the absence of DIF-1 cooperation. Alternatively, it cannot be excluded that most cell death/vacuolization in vivo is governed neither by DIF-1 nor by c-di-GMP. There might be other, as-yet-unidentified inducers of *Dictyostelium* cell death in vivo. Multiple inducers of the same cell death mechanism have been described in other models—for instance, in apoptotic cell death of animal cells.

Taken together, our results contribute to the clarification of induction of *Dictyostelium* cell death in vitro and of the DIF-1/c-di-GMP relationship in this case. It identifies some constraints on *Dictyostelium* cell death in vivo. It also suggests modifications of our mutational approach to find molecules involved in cell death in vitro, since the DIF-1 and the c-di-GMP pathways should converge at some point, downstream of which mutations should block both pathways. Such mutations could be selected by inducing cell death by both DIF-1 and c-di-GMP simultaneously. Would such mutations

also affect cell death in vivo? More generally, our results have implications for the relationships (phylogenetic, mechanistic) between the induction by c-di-GMP of *Dictyostelium* developmental cell death and the induction by c-di-GMP of innate immunity and other effects in animal cells. In addition, c-di-GMP has been incriminated in animal cell death (Karaolis *et al.*, 2005; Chandra *et al.*, 2014), and c-di-GMP can activate the inflammasome NLRP3 (Abdul-Sater *et al.*, 2013), which can lead to cell death (Willingham *et al.*, 2007). The implication of c-di-GMP in *Dictyostelium* cell death makes it tempting to investigate further its possible role in the death of cells other than those of *Dictyostelium*. A requirement for polyketides may be worth investigating in these and other c-di-GMP effects in animal cells.

## MATERIALS AND METHODS

### Handling of *Dictyostelium* cells, induction of development, and general molecular biology techniques

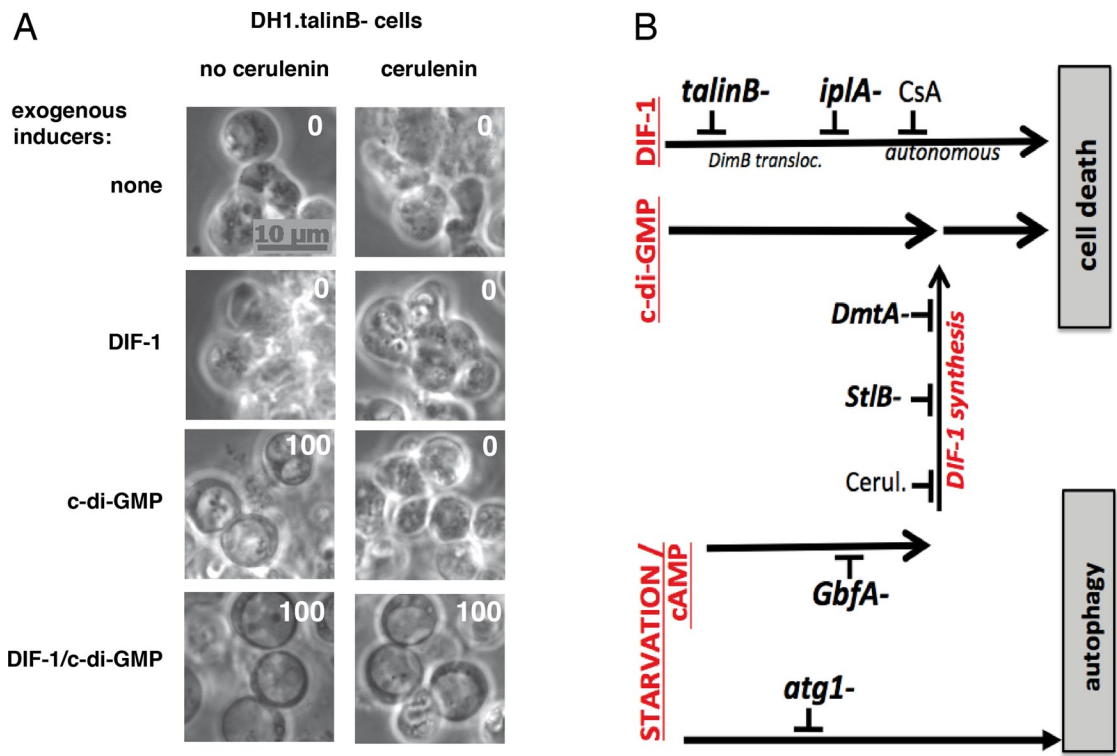
These were as described previously (Giusti *et al.*, 2008, 2009; Lam *et al.*, 2008).

### Induction and assessment of cell death as vacuolization

On the day before the experiment, cells were adjusted at  $3 \times 10^6$  cells/10 ml of HL5 medium/Falcon T25 flask. On the day of experiment, these exponentially growing cells were washed once in phosphate-buffered saline (Sørensen buffer [SB]) and incubated in SB containing 3 mM cAMP (Sigma-Aldrich, St. Louis, MO) for 8 h at 22°C in Lab-Tek culture chambers (155380; Nalge Nunc International, Rochester, NY) at a concentration of  $3 \times 10^5$  cells/ml/chamber. Cells were then washed in SB and incubated at 22°C in either SB alone or SB containing the differentiation factor DIF-1 (DN1000; Affiniti Research Products, Exeter, United Kingdom) at a final concentration of  $10^{-7}$  M, c-di-GMP sodium salt (C 057-01; Biolog, Bremen, Germany) at a final concentration of  $10^{-5}$  M, or combinations thereof or at other concentrations as indicated. After the indicated period of incubation, cells in the Lab-Tek chambers were examined using an Axiovert 200 microscope (phase contrast, oil immersion, 100 $\times$ ; Carl Zeiss, Jena, Germany) and photographed using an AxioCam MRC camera controlled by AxioVision 4.7 (Carl Zeiss). Images were subsequently homogeneously treated with Graphic Converter. Figures were assembled using Illustrator (Adobe Systems, San Jose, CA).

### Assessment of cell death as non-regrowth

Wild-type or mutant cells were incubated in Lab-Tek chambers as described. After a variable incubation period at 22°C, to initiate regrowth, 0.5 ml of SB was removed from and 1 ml of HL-5 was added to each Lab-Tek chamber. After 48–72 h of additional incubation at 22°C, vegetative cells resulting from regrowth were counted in a hemocytometer, and the results were graphed using Prism 6 (GraphPad, La Jolla, CA).



**FIGURE 5:** Recapitulation of some of the results on DIF-1 and c-di-GMP pathways inducing *Dictyostelium* cell death in monolayers. (A) Talin<sup>B-</sup> mutant cells were incubated or not with 50 μM cerulenin and with or without 100 nM DIF-1 and 10 μM c-di-GMP for 40 h. See the text for comments on the results. (B) Tentative representation of DIF-1 and c-di-GMP pathways inducing *Dictyostelium* cell death in vitro, with some of the mutations and drugs mentioned in this work. Bottom, a first signal—starvation plus cAMP—triggers autophagy and sensitizes cells to the second signal. Top, second signals would operate only on cells sensitized by the first signal. Exogenous DIF-1 triggers an autonomous pathway to cell death marked by several mutations. To induce cell death, exogenous c-di-GMP requires cooperation with endogenously synthesized (or exogenous, not represented) DIF-1. Middle, DIF-1 biosynthetic pathway, marked by other mutations, induced by cAMP, provides endogenous DIF-1 required for cooperation with exogenous c-di-GMP to induce cell death. We have not represented the *iplA*<sup>-</sup> mutation, which could inhibit both the exogenous autonomous DIF-1 pathway and exogenous DIF-1 cooperation.

### Preparation of DH1.GFP-DimB cells

Twenty million DHI cells were electroporated (3 μF; 1kV) with 10 μg of a GFP-DimB.BlastR vector expressing GFP-DimB controlled by the DimB promoter, kindly provided by Jeff Williams (University of Dundee, Dundee, United Kingdom), in 10 mM NaPO<sub>4</sub>, pH 6.1, and 50 mM sucrose in Volvic water and then cultivated in HL5 medium at 22°C for 24 h. Blasticidin (10 μg/ml) was then directly added on cells. After 8 d of blasticidin selection, cells were starved for 4 h, and the “greenest” cells (0.1% of the total population) were cloned by distributing (FACS Vantage; BD Biosciences, San Jose, CA) 1 cell/well in 96-well plates containing HL5 medium. Clones obtained after 7 d at 22°C were transferred to LabTek chambers, grown for 24 h, and then starved for 4 h in SB buffer plus 3 mM cAMP. Cells were washed, treated with 100 nM DIF-1 or 10<sup>-5</sup> M c-di-GMP for 5–10 min, and screened for nuclear translocation.

### Targeted mutagenesis of DgcA

The gene disruption vector pDgcA (Chen and Schaap, 2012) obtained from the Dicty Center (www.dictybase.org) was BamH1 linearized and then electrotransformed in DH1 cells. These were selected by blasticidin at 5 μg/ml and cloned by limiting dilution in wells of microtiter plates. Screening for clones with a deletion in the DgcA gene through plasmid integration by homologous recombination was by PCR. Primers were *dgkO1rAS* (GTAAATACCATCAATCAGTTTTGAC) and *dgkO2f-AS* (ATATTATCAGATGTTGTTTGT-

GTTTGTTTC), yielding a 0.35-kb product only in the wild type, and pUCf (ACGCAATTAATGTGAGTTAGCTCACTC) and DgcA-1549-AS (CAACAACATTTATTTGACTAATTCCTTTTTTC), yielding a 0.87-kb product only upon DgcA mutation.

### Targeted mutagenesis of stlB

To create a *stlB* gene insertion construct, a *stlB* DNA fragment was amplified by PCR from genomic DNA nucleotide (nt) 6458 to nt 8295, using primer pairs *stlB* 6458/8295 bearing *HhaI* restriction sites, and then cloned into pGEMT-easy vector. After plasmid linearization by *BsaBI* (at nt 7416), a blasticidin resistance cassette removed from plasmid pLBP was inserted into this fragment. The insertion plasmid was confirmed by sequencing and then digested with *HhaI*. The fragment was purified from agarose gel and introduced into DH1 cells by electroporation. Cells were distributed into 96-well plates immediately (7 × 10<sup>4</sup> cells/well). After selection with blasticidin at 10 μg/ml and recloning, transformed clones were screened by PCR using primer pairs *stlB*-6370/Bsr5 and *stlB*-6370/Bsr8922. These primers should yield 1.2- and 4.1-kb products, respectively, in an insertion mutant, whereas in wt cells they should yield no product and a 2.5-kb product, respectively. Primer sequences were *stlB*-6458/*HhaI*, GCGCATCATTGAGTTGTTTCATTTACC; *stlB*-8295/*HhaI*, GCGCAACTCTTTCACCTGCATCC; *stlB*-6370, AGCCATTAGATTCATGTCATCCGGT; *stlB*-8922, GCGCACCACCAGGATGAGTAGCAAAG; and Bsr5, Bsr cassette, CGCCAACCAAGTTTTTTAAACC.



## Targeted mutagenesis of DmtA

To create a *DmtA* gene insertion construct, a *DmtA* DNA fragment was amplified by PCR from genomic DNA nts 540–1168, using primer pairs *DmtA A/AS* bearing *HhaI* restriction sites, and then cloned into pGEMT-easy vector. After plasmid linearization by *BsaBI* (at nt 555), a blasticidin resistance cassette removed from plasmid pLBP was inserted into fragment. The insertion plasmid was confirmed by sequencing and then digested with *HhaI*. The fragment was purified from agarose gel and named *DmtAins* construct. This construct was introduced into DH1 cells by electroporation. Cells were distributed into 96-well plates immediately ( $7 \times 10^4$  cells/well). After selection with blasticidin at 10  $\mu\text{g/ml}$  and recloning, transformed clones were screened by PCR using primer pairs *Bsr5/DmtA1958* and *DmtA323/DmtA1958*. These primers should yield 1.6- and 3.2-kb products in an insertion mutant, respectively, whereas in wt cells they should yield no product and a 1.6-kb product, respectively. Primer sequences were *DmtA-S*, GCGCAGATGGTACCAAAGTGTGTGCAT (*HhaI*); *DmtA-AS*, GCGCAGACATTCTTTACTATCTGGAAGG (*HhaI*); *Dmt-323*, CTGATAGCTGGGTTAA-CAATGTGTA; *DmtA-1958*, TCATTTTGTCCAATCACTCAAGGT; and *Bsr5*, CGCCAACCCAAGTTTTTTTAAACC.

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