

# NucA is required for DNA cleavage during transformation of *Bacillus subtilis*

Roberta Provvedi,<sup>†</sup> Inês Chen and David Dubnau\*  
Public Health Research Institute, 455 First Avenue, New  
York 10016, USA.

## Summary

We have re-examined the roles of *nucA* and *nin*, in the transformation of *Bacillus subtilis* as conflicting accounts have been presented concerning the importance of these genes for transformation. The present report demonstrates that *nucA* deficiency lowers the rate of DNA transport and that NucA is needed for the double-strand cleavage of transforming DNA, probably acting directly as an endonuclease. A relative paucity of DNA termini, resulting from the absence of this endonuclease activity, most probably accounts for the decreased transport rate. NucA is a bitopic integral membrane protein, with its C-terminus external to the membrane where it is appropriately located to effect the cleavage of bound transforming DNA. We have also investigated the roles of the known competence genes in the DNA processing that accompanies transformation in *B. subtilis*. The genes that are required for DNA transport (*comEA*, *comEC* and *comFA*) are also required for the degradation of the non-transforming strand that accompanies internalization, but *comEC* and *comFA* are not needed for the double-strand cleavage that occurs external to the cell membrane.

## Introduction

Genetic transformation in *Bacillus subtilis* depends on a set of proteins whose expression is controlled by the competence transcription factor ComK. These proteins mediate DNA binding and uptake and include the products of the *comG*, *comC*, *comF* and *comE* loci (reviewed in Dubnau, 1999).

Upon binding to the competent cell surface, transforming DNA first undergoes limited double-strand cleavage. After this, one strand is internalized whereas the other (non-transforming) strand is degraded to yield phosphorylated, acid-soluble products which are released into the

medium (Dubnau and Cirigliano, 1972a). In *Streptococcus pneumoniae*, transforming DNA is processed similarly. In this organism the EndA protein is required for transport and for degradation of the non-transforming strand but not for DNA binding to the cell surface (Lacks and Neuberger, 1975; Lacks *et al.*, 1975). *Bacillus subtilis* lacks an *endA* orthologue, and no other transport nuclease has been identified.

However, a *B. subtilis* protein with nuclease activity has been characterized that may play a role in transformation. *nucA* and *nin* encode 16.5 and 18 kDa proteins respectively (Vosman *et al.*, 1988) and expression of the *nucA-nin* operon was shown to require ComK, the competence transcription factor (van Sinderen *et al.*, 1995a), which binds to the region upstream of *nucA* (van Sinderen *et al.*, 1995b). NucA possesses a manganese-stimulated DNase activity and Nin appears to act as an inhibitor of NucA (Vosman *et al.*, 1988; van Sinderen *et al.*, 1995b). Inactivation of the *nucA-nin* locus was initially reported to cause a moderate decrease in transformability (Vosman *et al.*, 1988). However, a more recent study failed to replicate this finding (van Sinderen *et al.*, 1995b) and the role of NucA during transformation remained uncertain.

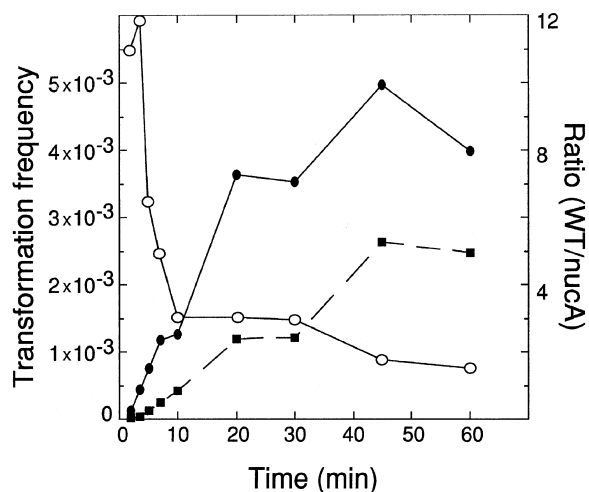
We have reinvestigated the roles of *nucA* and *nin*, as well as those of other known competence genes, in transformation and in DNA cleavage and degradation during transformation. We demonstrate that NucA acts as an endonuclease for double-strand DNA cleavage on the cell surface during transformation and that it is localized as an integral membrane protein. We further demonstrate that the DNA transport proteins ComEC and ComFA are not required for double-strand cleavage. These results are used to propose an updated version of the transformation pathway.

## Results

### *nucA* and *nin* play individual roles during transformation

We first constructed a deficient mutant in which the chromosomal *nucA* gene was disrupted by insertion of a spectinomycin cassette (see *Experimental procedures*). The transformation efficiency of this strain (BD2941) was analysed in a time course experiment. Cells grown to competence were incubated with chromosomal DNA and samples taken at various times were treated with DNase and plated to determine the transformation frequency

Accepted 12 February, 2001. <sup>†</sup>Present address: Sezione di Microbiologia, Dipartimento di Biologia Molecolare, Università di Siena, 53100 Siena, Italy. \*For correspondence. E-mail dubnau@phri.nyu.edu; Tel. (+1) 212 578 0842; Fax (+1) 212 578 0804.



**Fig. 1.** Transformation kinetics of the *nucA* mutant. BD2941 (*nucA*, ■) and the isogenic wild-type strain (BD630, ●) were incubated with DNA and samples were treated with DNase at the indicated times before plating to determine the transformation frequency. Selection was for leucine prototrophy. The ratio of wild-type to *nucA* transformation is also shown (○).

(Fig. 1). Because treatment with DNase removes unbound DNA, as well as DNA that is exposed on the cell surface, this method only detects transformants that are derived from DNA that has been internalized at the time of DNase addition. Figure 1 shows that the *nucA* mutant exhibits a decreased rate of transformation. During the first 7 min in this experiment, the rate was 5- to 12-fold lower than that exhibited by the isogenic wild-type strain. After 45 min, the level of transformation in the mutant reached a level that was less than twofold lower than that of the wild type. We conclude from these data and from other similar experiments that *nucA* is not absolutely required for transformation but that in its absence the rate of DNA internalization is decreased, with a relatively minor effect on the final transformation frequency.

Sequence analysis of the *nucA*-coding region identified a downstream open reading frame (ORF), *nin* (Vosman *et al.*, 1988). *nucA* and *nin* belong to the same transcription unit, and it has been reported that the transformation frequency of a *nin* mutant was decreased fourfold. To determine whether the *nucA* phenotype displayed in Fig. 1 was due to a polar effect on *nin*, we first constructed a *nin* deficient mutant in the BD630 background and confirmed that the transformation frequency of this strain (BD2942) was reduced nearly sevenfold (Table 1). We then expressed an intact copy of *nin* in the *nucA* deficient background. To accomplish this, the *nin*-coding sequence was cloned into pG67 (Chung and Dubnau, 1998) which placed it under control of the competence-dependent *comG* promoter and permitted its integration at the ectopic *amyE* locus. In

**Table 1.** Complementation test with *nin* and *nucA* mutants.

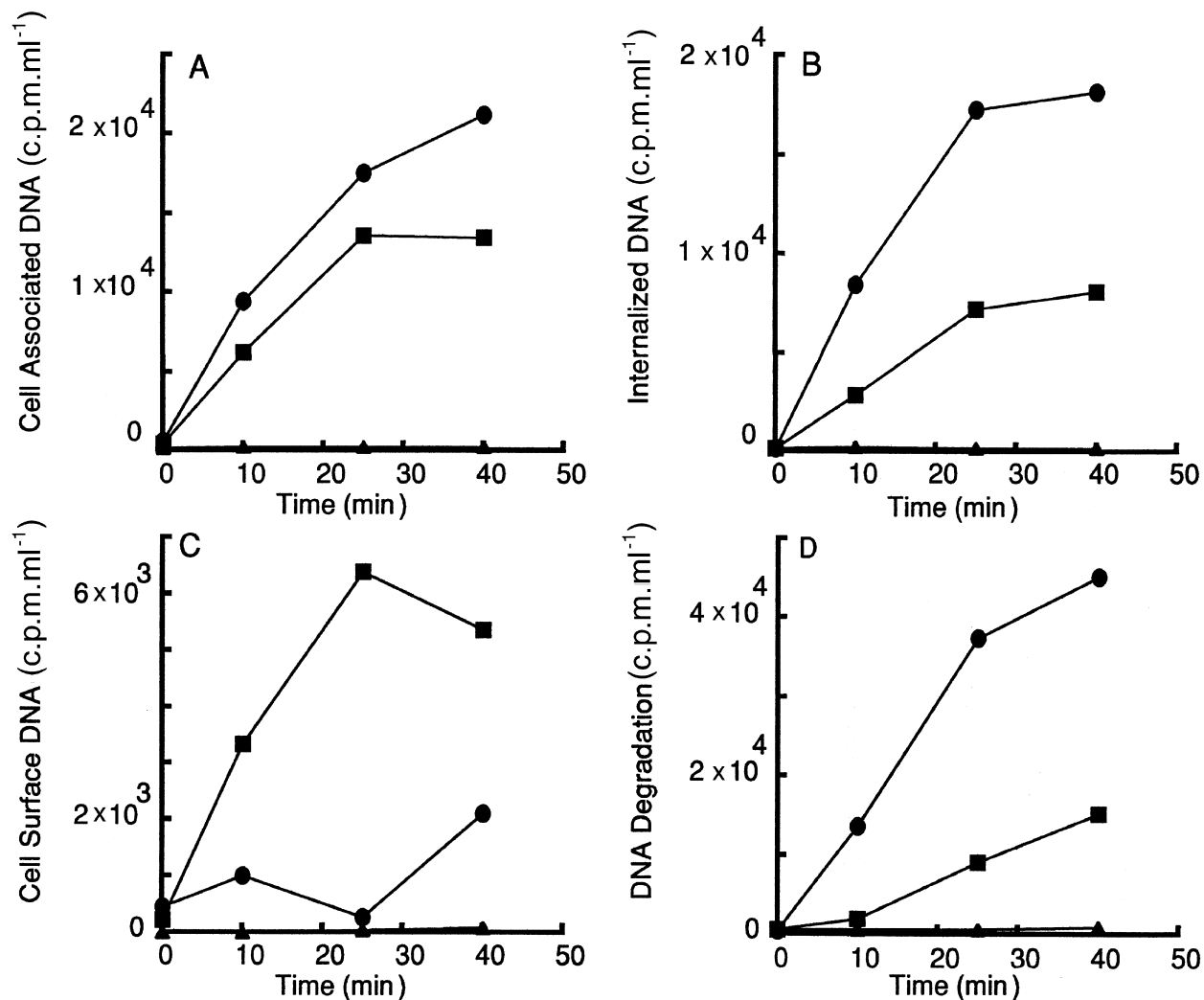
Strain	Relevant genotype	Transformation frequency <sup>a</sup>
BD630	Wild-type	1
BD2941	<i>nucA</i>	0.08
BD2942	<i>nin</i>	0.15
BD3007	<i>nucA amyE::nin</i>	0.08
BD3005	<i>nin amyE::nin</i>	0.7

**a.** Transforming DNA was incubated with the individual strains for 10 min before the addition of DNase and plating for leucine prototrophy. Data were normalized to the BD630 value, which was 0.3%.

the resultant strains the expression of *nin* at the *amyE* locus did not complement the *nucA* mutation for transformation (strain BD3007), whereas it could restore transformation to nearly the wild-type level in a *nin*-deficient mutant (strain BD3005) (Table 1). These results demonstrate that *nucA* and *nin* play individual roles in transformation.

#### *The nucA* mutant accumulates DNA on the surface during transformation

Radiolabelled DNA was used to determine the relative extents of DNA binding, internalization (transport) and degradation by wild-type and *nucA*-deficient cultures grown through the competence regimen (Fig. 2). In these assays, [<sup>3</sup>H]-DNA was added to cell cultures grown to competence and samples were processed at various times. The radioactivity associated with the cells after centrifugal washing was used as a measure of surface bound plus internalized DNA, and the amount of radioactivity that persisted after DNase treatment was used to determine transport (Dubnau and Cirigliano, 1972b). Surface bound DNA was determined as the difference between the total [<sup>3</sup>H]-DNA associated with the cells and the DNase-resistant uptake. Degradation was measured as the amount of radioactivity recovered from the supernatant after TCA precipitation of the cell-associated [<sup>3</sup>H]-DNA and the unbound [<sup>3</sup>H]-DNA present in the culture medium. This degradation, which occurs on the cell surface, is due to the action of an unknown nuclease on the non-transforming strand. The products of this nuclease are released into the extracellular medium (Dubnau and Cirigliano, 1972a). As a negative control we used an isogenic *comK* strain that is completely competence deficient. Figure 2 demonstrates that DNA transport (Fig. 2B) and degradation (Fig. 2D) were decreased in the *nucA* mutant. Figure 2C reveals that the DNA associated with the cell surface was considerably increased in the *nucA* mutant. We conclude from this that the decreased rate of transformation of the *nucA* mutant (Fig. 1) is due to the slower DNA transport demonstrated in Fig. 2B. The



**Fig. 2.** DNA binding, degradation and internalization. <sup>3</sup>H-thymidine radiolabelled DNA was used to transform BD2941 (*nucA*, ■), the isogenic wild-type strain (BD630, ●) and a non-transformable *comK* strain (BD2255, ▲). The total cell associated radioactivity (A), internalized DNA (B), cell surface associated DNA (C) and DNA products released as acid soluble radioactivity (D) were determined as a function of time.

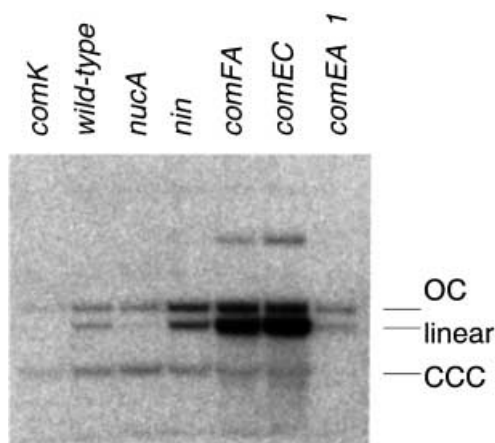
total cell-associated DNA was somewhat lower in the *nucA* mutant (Fig. 2A), although this was not observed in other experiments. The *nin* mutant was also tested for DNA binding, uptake and the formation of acid-soluble products. No significant differences were found compared with the wild type (data not shown). We next addressed the cause of the decreased rate of DNA transport in the *nucA* mutant.

#### *nucA* is required for DNA cleavage

At least two nuclease activities are required during transformation. The first introduces double-strand cuts in DNA prior to transport. This event, which we refer to as cleavage, occurs outside the cell membrane (Dubnau and Cirigliano, 1972a). The second activity degrades the

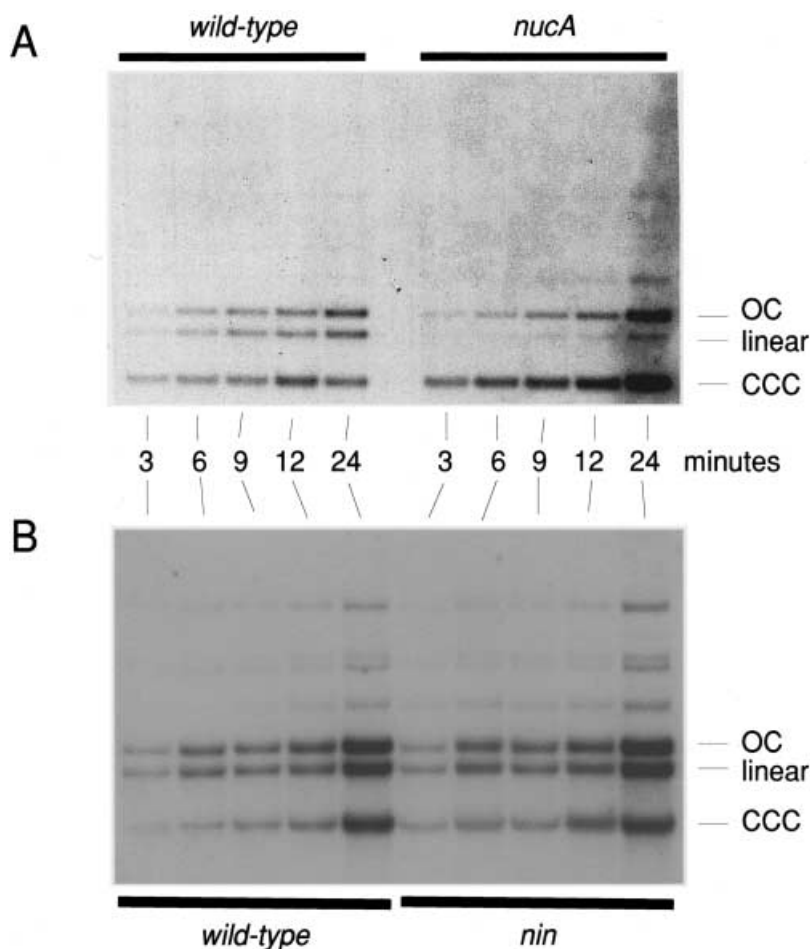
non-transforming strand during uptake, and is referred to here as degradation. We entertain two hypotheses concerning the role of *nucA*, which is known to exhibit nuclease activity *in vitro* (Mulder and Venema, 1982a, b; Smith *et al.*, 1983, 1984, 1985; Vosman *et al.*, 1988; van Sinderen *et al.*, 1995b). According to the first hypothesis, NucA may be the enzyme responsible for cleavage, and the decreased rate of internalization in the *nucA* mutant may simply be due to a paucity of DNA termini available as substrates for the transport machinery. A second possibility is that NucA is needed for degradation of the non-transforming strand and that the decreased rate of transport results from the absence of this degradation.

To determine whether NucA plays a role in cleavage, we incubated competent cells with covalently closed circular (CCC) pBluescript DNA for 8 min, and checked



**Fig. 3.** Linearization of plasmid DNA by competent cells. The wild-type and mutant strains indicated were incubated for 8 min with pBluescript DNA and the DNA associated with the cell surface was recovered as described in *Experimental procedures*. The DNA was resolved on an agarose gel, which was blotted and probed with  $^{32}\text{P}$ -labelled pBluescript DNA. Uncleaved and linearized (with *Sma*I) pBluescript DNA were included in the gel for comparison, and the positions of the various plasmid forms referred to in the text are indicated.

for the presence of plasmid linear forms recovered from the cell surface (Fig. 3). For this experiment the wild-type strain was compared with *nucA*, *nin*, *comFA* and *comEC* mutants. In addition, we used a mutant with an in frame deletion in *comEA* (*comEA* $\Delta$ 1) that removes 41 residues from the central portion of the ComEA protein (from a total of 205 residues in the intact protein). These last three mutants are able to bind DNA but cannot carry out transport, and are therefore transformation deficient (Hahn *et al.*, 1987, 1993; Londoño-Vallejo and Dubnau, 1994a; Inamine and Dubnau, 1995). The *nucA*, but not the *nin* mutation, decreased the amount of linearized plasmid DNA on the cell surface (Fig. 3). In contrast, the *comFA* and *comEC* null mutants exhibited strong linear and open circular (OC) signals. This corresponds to the accumulation of DNA on the cell surface of these mutants, which are incapable of transport. The diffuse signal detected below the linear DNA in these two strains is due to the presence of lower molecular weight forms produced by multiple cleavage events on a given plasmid molecule. These products remain associated with the cell surface in the absence of internalization, and are not seen



**Fig. 4.** Kinetics of DNA linearization by wild-type, *nucA* (BD2941, A) and *nin* (BD2942, B) competent cells. The cells were incubated with pBluescript DNA for the times indicated and the cell surface DNA was recovered as described in *Experimental procedures*. The DNA was resolved on an agarose gel, which was blotted and probed with  $^{32}\text{P}$ -labelled pBluescript DNA.

in the other strains, presumably because they have less total DNA on the cell surface and because these strains transport DNA, leaving less time for multiple cleavage events to occur. At least one higher form, corresponding to linear dimer, is clearly visible in the *comFA* and *comEC* mutants. In the *comEAΔ1* mutant, almost all the CCC DNA bound to the surface is converted to the linear form; most of the OC signal in this sample (as in the wild-type and *nucA* mutant) probably represents nicked DNA pre-existing in the pBluescript sample. The lower total amount of cell surface-associated DNA in the *comEAΔ1* mutant is consistent with our previous report that this mutation reduces DNA binding (Inamine and Dubnau, 1995). Although in Fig. 3 there appear to be elevated amounts of both linear and OC forms in the *nin* mutant, these differences were not observed in other experiments (see Fig. 4). It appears that of the mutations tested, only the *nucA* mutation decreased the formation of linear DNA.

The results obtained with the *nucA* and *nin* mutants were confirmed and extended in time course experiments (Fig. 4A and B respectively). Plasmid linearization was markedly delayed in the *nucA* mutant. Inspection of Fig. 4A, as well as quantitative analysis of the results with a phosphorimager (not shown), confirmed that more total DNA is accumulated on the surface of the *nucA* deficient mutant compared with the wild type, consistent with the results of Fig. 2C. Again no difference was detected between wild-type and *nin* deficient strains.

#### *The nucA phenotype is molecular weight dependent*

If the primary role of NucA is to generate DNA termini appropriately positioned for engagement with the transport machinery, we might expect that transformation with lower molecular weight DNA would decrease the dependence of transformation on the NucA protein. This expectation was fulfilled. For instance in one experiment, chromosomal DNA was cleaved with a mixture of *SmaI* and *BalI*. This combination of enzymes is predicted to generate a 3.5 kb fragment carrying the *metB* gene. This DNA, and also uncut chromosomal DNA, were used to transform competent cultures of the wild-type and *nucA* mutant. After incubation for 10 min, the reactions were stopped by the addition of DNase and the frequencies of transformation to methionine prototrophy were determined. With the uncut DNA, the frequency of transformation of the mutant strain was 11-fold lower than that of the wild type ( $6.0 \times 10^{-4}$  vs.  $6.7 \times 10^{-3}$ ). When the cleaved DNA was used, identical reduced transformation frequencies were obtained with both the wild-type and mutant strains ( $2.4 \times 10^{-7}$ ). It appears, therefore, that the effect of the *nucA* null mutation was bypassed by the artificial introduction of double-strand cleavages.

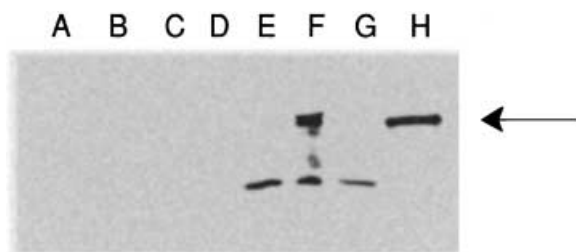
Several conclusions may be drawn from these

experiments. As proposed above by the first hypothesis, NucA is required for DNA cleavage on the cell surface, probably acting directly as a nuclease. The reduced degradation noted in the *nucA* mutant (Fig. 2D) is easily explained as a consequence of the reduced rate of transport. Finally, it appears that *Nin*, *ComFA* and *ComEC* are not needed for cleavage. Because only an in frame *comEA* mutant could be tested, we cannot conclude that *ComEA* plays no role in cleavage.

#### *NucA is an integral membrane protein with its C terminus outside*

NucA is predicted to contain a single transmembrane segment near the N-terminus (residues 5–24) and a possible signal peptidase cleavage site between residues 31 and 32 (Nielsen *et al.*, 1996). To address the localization and topology of NucA, we constructed fusions that attached the *Escherichia coli* proteins PhoA and LacZ in frame with the C-terminus of NucA. These fusions were integrated by Campbell-like recombination at *nucA*, placing them under control of the *nucA* competence-driven promoter. The alkaline phosphatase and  $\beta$ -galactosidase activities of the two fusion strains were determined, as well as those of the parent strain lacking the fusions (BD630), after growth through the competence regimen. Although these strains were grown in medium containing 125 mM phosphate to minimize the endogenous alkaline phosphatase activity, a low basal activity was detected (0.026 units). The activity of the *nucA-phoA* fusion strain was 21-fold higher than this, suggesting that the C-terminus of NucA is external to the membrane. The  $\beta$ -galactosidase activities of the wild-type and *nucA-lacZ* strains were 0.011 and 0.024 units  $\text{mg}^{-1}$  protein. This small difference was probably not significant and the low  $\beta$ -galactosidase activity could not be explained by the presence of a low amount of the NucA–lacZ fusion protein. Using a monoclonal antibody against LacZ, the NucA–LacZ fusion signal was clearly visible in Western blots (Fig. 5). For comparison, we used a LacZ fusion to a cytoplasmic portion of *ComEC* (I. Draskovic and D. D., unpublished results). When comparable amounts of purified membranes from the *comEC-lacZ* and *nucA-lacZ* strains were applied to the gels, the *nucA* fusion gave a clear signal whereas no *comEC* signal was detectable. In spite of this, the  $\beta$ -galactosidase specific activity of the *ComEC*–LacZ fusion strain was fivefold above the endogenous level. Because only the PhoA fusion exhibited an enzyme activity significantly above background, we conclude that the C-terminus of NucA is external to the membrane in competent cells.

We next determined whether NucA is membrane localized, as predicted, and whether it is an integral or peripherally associated membrane protein. In Western



**Fig. 5.** Membrane localization of NucA. Subcellular fractions were prepared from the *nucA-lacZ* fusion strain (BD3004) and from the wild-type isogenic parent strain (BD630), fractionated by SDS-PAGE, blotted and probed with anti-LacZ antiserum. The following fractions were analysed. A, BD630 cytoplasm; B, BD3004 cytoplasm; C, BD630, cell wall; D, BD3004, cell wall; E, BD630 membrane; F, BD3004, membrane; G, BD3004, NaOH soluble fraction; H, BD3004, insoluble fraction. The position of the NucA-LacZ signal is indicated by an arrow. A cross-reacting membrane protein is also visible in the blot.

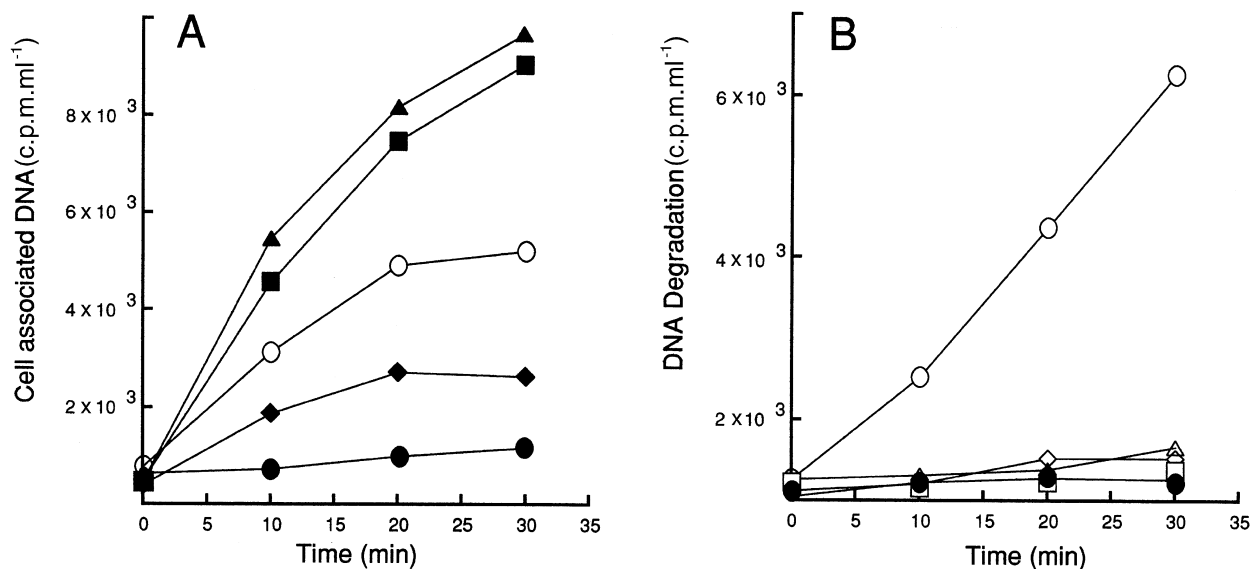
blots using monoclonal antibody against LacZ, no NucA-LacZ signal was detected in the cytosolic or cell wall fractions (Fig. 5). A strong signal was observed associated with the membrane, and this signal was insoluble in 0.1 N NaOH (Russel and Model, 1982). No Western blot signal was detected in any of the fractions from the wild-type parent lacking the fusion construct. Similar results were obtained with the NucA-PhoA fusion using antibody directed against PhoA (not shown). This localization of NucA as an integral membrane protein, and the disposition of its C-terminus external to the membrane is consistent with its proposed role as a DNA-cleaving enzyme, because cleavage occurs outside the membrane (Dubnau and Cirigliano, 1972a, 1974; Dubnau, 1976).

### Mutations interfering with DNA transport prevent degradation

Three gene products have been shown to play essential roles in the internalization of DNA: ComFA, ComEC and ComEA. ComEA acts as the receptor for double-strand DNA binding (Provvedi and Dubnau, 1999), whereas ComFA and ComEC play no role in binding (Hahn *et al.*, 1987; Londoño-Vallejo and Dubnau, 1994a, b; Inamine and Dubnau, 1995). We determined whether these proteins are needed for the degradation of the non-transforming strand during DNA internalization. Figure 6 demonstrates that degradation does not occur in the three transport mutants. As expected, the *comEC* and *comFA* transport mutants accumulate more DNA on the cell surface than the wild-type strain, because they fail to transport and degrade the non-transforming strand. The *comEA* $\Delta 1$  mutant does not show this enhanced accumulation because it binds less DNA than the wild-type strain.

### Discussion

Previous work has described a transformation pathway for *B. subtilis* (reviewed in Dubnau, 1999). DNA is bound to the cell surface of competent cells at a limited number of sites and at few points on each DNA molecule, resulting in DNA-cell complexes in which the bulk of each DNA molecule is extended into the aqueous medium. After binding, the DNA undergoes double-strand cleavage events, presumably at the points of attachment to the cell surface. One strand is then transported to the interior



**Fig. 6.** DNA binding and degradation in transport mutants. Total cell associated  $^3\text{H}$ -thymidine DNA (A) and DNA products released as acid soluble radioactivity (B) were measured in wild-type (BD630, ○), *comFA* (BD1238, ▲), *comEC* (BD1247, ■), *comEA* $\Delta 1$  (BD2327, ◆), and *comK* (BD2255, ●) strains as a function of time.

of the cell and its complement is degraded, with release of the acid soluble products to the medium.

Models for DNA uptake invoke the existence of water-filled pores, with associated proteins mediating DNA binding, fragmentation, degradation and internalization. About a dozen genes that encode proteins required for transformation have been identified, and the expression of all of them is dependent on the competence transcription factor, ComK. The roles of several of these proteins have been established or are strongly suggested by the available data. For instance, the integral membrane protein ComEA is a DNA receptor for transformation (Provvedi and Dubnau, 1999). It binds non-specifically to double-stranded DNA using an extracellular DNA-binding domain. The seven proteins encoded by the *comG* operon are also needed for DNA binding to the cell, but they do not appear to be DNA-binding proteins. Instead, they are needed to provide access of transforming DNA to the ComEA receptor, which in the absence of the ComG proteins is obscured by the cell wall (Provvedi and Dubnau, 1999). The ComG proteins may, therefore, remodel the wall or establish a channel that traverses the wall. ComC is a specific peptidase needed for processing the membrane localized ComG pilin-like proteins, and for this reason is also required for DNA binding (Chung *et al.*, 1998; Chung and Dubnau, 1995, 1998). ComEC is a polytopic integral membrane protein with a structure suggestive of a channel-forming protein and is required only for DNA transport. ComFA is probably directly involved in translocating DNA across the cell membrane as it resembles known ATP-dependent DNA helicases and translocases (Londoño-Vallejo and Dubnau, 1993). Like ComEC, it behaves like an integral membrane protein, with its bulk located in the cytosolic compartment.

In the present report we have addressed several questions concerning the transformation pathway. First, we have inquired as to the roles of the NucA and Nin proteins, which are under ComK control. NucA exhibits a manganese-stimulated DNase activity associated with membrane vesicles of *B. subtilis* grown to competence, whereas Nin acts as an inhibitor of NucA (Vosman *et al.*, 1988; van Sinderen *et al.*, 1995b). Because conflicting reports exist concerning the possible roles of these two proteins in processing nucleic acids during transformation, we have re-examined the phenotypes of *nucA* and *nin* mutants. It should be noted that the dependence of *nucA* and *nin* expression on ComK does not prove that these genes are required for transformation, as other ComK-dependent genes are not involved in transformation (J. Hahn *et al.*, unpublished results). Our present results suggest that NucA is the enzyme responsible for the double-strand cleavage of DNA on the cell surface, as the effect of a null mutation in *nucA* is to severely

decrease the rate at which cleavage occurs (Figs 3 and 4). This in turn decreases the rates of DNA transport and degradation (Fig. 2). Figure 3 also demonstrates that the ComFA and ComEC transport proteins, as well as the central portion of ComEA, are not required for cleavage. Although only a partially deleted ComEA protein could be tested, because the null mutant binds no DNA, this protein may act to position the DNA substrate near the active site of NucA.

Because a strong effect on the frequency of DNA cleavage affects the rate of internalization more than the final yield, it appears that the cleavage event is not absolutely essential for DNA transport. It is likely that the role of cleavage is to provide DNA termini as substrates for internalization. Kinetic analysis has demonstrated that the cleavage products are normally precursors of internalized DNA (Dubnau and Cirigliano, 1972b). We believe that in the absence of cleavage, transport depends on a time-dependent search for pre-existing termini, as in high molecular weight DNA in solution, the termini would be shielded from contact with the ComEA receptors much of the time by internal DNA segments, until they are exposed by thermal motion. In lower molecular weight DNA, this shielding effect will be diminished. This hypothesis explains two aspects of the *nucA* phenotype: the dependence of this phenotype on the initial molecular weight of the transforming DNA and the decreased rate of internalization. However, we cannot at present exclude the alternative hypothesis that transport is completely dependent on newly introduced termini, and that these are introduced by a back-up nuclease in the *nucA* mutant. Our results provide two likely explanations for the conflicting reports concerning the role of NucA in transformation. Because the rate of DNA internalization is affected more than the final yield of transformants, the results obtained depend critically on when transformation is measured. and if the initial molecular weight of the transforming DNA used were low, the role of NucA would be obscured. Our *phoA* and *lacZ* fusion results suggest that NucA is an integral membrane protein with its C-terminus outside the membrane (Fig. 5). This is consistent with the suggested role for this protein, as the DNA cleavage products are also external to the membrane (Dubnau and Cirigliano, 1972a).

In the absence of Nin, the transformation frequency was decreased about sevenfold, in agreement with a previous report (van Sinderen *et al.*, 1995b), and confirming a role for Nin in transformation. Nin deficiency did not detectably affect DNA binding, cleavage, transport or degradation. Because Nin acts *in vitro* to limit the nuclease activity of NucA (Vosman *et al.*, 1988; van Sinderen *et al.*, 1995b) it is possible that in its absence, the average size of the internalized DNA fragments is suboptimal, thereby reducing the efficiency of integration.

A puzzling fact concerns the interaction of Nin and NucA. It is certain that NucA acts outside the membrane. However, the sequence of Nin, which exhibits hydrophilicity throughout its length, does not suggest that it is secreted or localized in the membrane. Although Nin and NucA seem to interact *in vitro* (Vosman *et al.*, 1988; van Sinderen *et al.*, 1995b) it is not clear how this interaction would occur *in vivo*. One possibility is that Nin is secreted by a special mechanism. Another is that it interacts with the N-terminus of NucA in the cytosol. A third attractive alternative is that Nin serves to protect chromosomal DNA from cleavage by NucA prior to externalization of this nuclease. In a *nin* mutant, viability of the competent cells would be compromised. Because only 5–10% of the cells in a culture are competent, this would be reflected in a loss of transformants but not in the total viable count, as observed. In any event it is clear that Nin, and the transport proteins ComFA and ComEC, are not involved in the double-strand cleavage of transforming DNA (Fig. 3). These results are reasonable, as transport follows the cleavage event.

We have examined the roles of the transport proteins in the degradation of the non-transforming strand (Fig. 6). Null mutations in *comFA* and *comEC* prevent this degradation, as does the in frame deletion in *comEA* (*comEAΔ1*). We have suggested that ComEA acts to present bound DNA to the transport apparatus, and that the ComEAΔ1 protein cannot carry out this presentation function (Provvedi and Dubnau, 1999). This mutation apparently prevents the bound DNA from gaining access to the degradative nuclease, but does not prevent access to NucA. Why does elimination of either ComEC or ComFA prevent degradation? Although it may be argued that one of them is actually the degradative nuclease, why are both needed for degradation? One possibility is that these proteins are needed for the integrity of a supramolecular complex that is required for both degradation and transport. Another is that transport is needed to present an incoming DNA terminus to the degradative nuclease. A result with the *comFAΔS1* in frame deletion is consistent with the first hypothesis (not shown). This mutation

reduces transport to the level observed in a *comFA* null mutant (Londoño-Vallejo and Dubnau, 1993). Degradation, however, still proceeds at about 30% of the rate observed in the wild-type strain. Perhaps the ComFAΔS1 mutant protein is sufficient to maintain the proposed supramolecular complex in a form capable of exhibiting partial degradative activity. Also consistent with the presence of ComFA in such a complex is the dominant negative phenotype of certain *comFA* point mutations (Londoño-Vallejo and Dubnau, 1994b).

The present view of the early steps in transformation in *B. subtilis* can be summarized as follows. Double-strand DNA is first bound to the cell surface by interaction with the C-terminal domain of ComEA. This protein is accessible to the external medium via interruptions in cell wall integrity introduced by the ComG proteins. The bound DNA is cleaved by NucA, which like ComEA is anchored to the membrane with its C-terminus extended to the outside. The cleavage products presumably remain attached to ComEA, which then acts to present a newly formed DNA terminus to the transport apparatus, composed of ComFA and ComEC. In the absence of NucA, this process will occur more slowly, as ComEA must bind near a pre-existing DNA terminus. This suggests that a cyclical search process of DNA binding and release may occur, permitting the eventual location of a terminus. This is plausible, as the binding affinity of ComEA for DNA is moderate ( $K_d \sim 500$  nM) (Provvedi and Dubnau, 1999).

## Experimental procedures

### Bacterial strains

All strains used in this work were derivatives of *B. subtilis* 168 and are described in Table 2. *Escherichia coli* strains are derivatives of JM109 or XL-1 Blue and were grown in Luria-Bertani (LB) medium with ampicillin ( $100 \mu\text{g ml}^{-1}$ ). Transformation of *B. subtilis* was carried out as described previously (Albano *et al.*, 1987). In the time course experiments with BD2941,  $100 \mu\text{g ml}^{-1}$  DNase was added to stop DNA internalization.

**Table 2.** Strains.

Strain	Genotype	Origin
BD630	<i>his leu met</i>	–
BD2255	<i>his leu met comK</i> (Tn10 in promoter, <i>cam</i> ) <i>amyE::comGA-lacZ</i> (Km)	Luttinger <i>et al.</i> (1996)
BD2327	<i>his leu met comEAΔ1</i>	Inamine and Dubnau (1995)
BD2941	<i>his leu met nuca</i> (Sp)	This work
BD2942	<i>his leu met nin</i> (Sp)	This work
BD3003	<i>his leu met nuca-phoA</i> (Cm)	This work
BD3004	<i>his leu met nuca-lacZ</i> (Cm)	This work
BD3005	<i>his leu met nin</i> (Sp) <i>amyE::nin</i> (Cm)	This work
BD3007	<i>his leu met nuca</i> (Sp) <i>amyE::nin</i> (Cm)	This work
BD1247	<i>his leu met comEC518</i> (Tn917 <i>lacZ</i> in <i>comEC</i> , Em)	Hahn <i>et al.</i> (1993)
BD1238	<i>his leu met comFA</i> (Tn917 <i>lacZ</i> , Em)	Londoño-Vallejo and Dubnau (1993)

### Construction of *nucA* and *nin* null mutants

The *nucA* null mutation was constructed as follows. The entire *nucA* was amplified by PCR and cloned in pUCCM18, a pUC18 derivative containing a chloramphenicol resistance ( $\text{Cm}^R$ ) gene (Inamine and Dubnau, 1995), using the restriction sites *KpnI* and *BamHI*. The *nucA*-coding frame was then interrupted by insertion of a spectinomycin (*Sp*) cassette at a *BalI* site. This cassette was derived from plasmid pIC156 after cleavage by *SmaI* (Steinmetz and Richter, 1994). The resulting plasmid was named pRP7. The *nucA*-deficient mutant (BD2941) was obtained by transforming competent *B. subtilis* strain BD630 cells with *Scal*-linearized pRP7 DNA, with selection for  $\text{Sp}^R$  and screening for  $\text{Cm}^S$  transformants in which the *nucA* gene was disrupted by the insertion of the *Sp* cassette (as confirmed by PCR analysis). A similar procedure was followed to obtain the *nin* mutant. A PCR product containing all of *nin* was cloned in pBluescript KS<sup>-</sup> after cleavage by *BamHI* and *KpnI* and subsequently the *nin*-coding frame was interrupted by inserting the *Sp* cassette at the *EcoRI* site within the *nin* ORF. The resulting plasmid (pRP8) was linearized with *Scal* and used to transform *B. subtilis* BD630 cells with selection for  $\text{Sp}^R$  transformants. Again, disruption of *nin* was confirmed by PCR analysis.

To express *nin* under competence control at an ectopic locus in *nucA* and *nin* backgrounds (BD3007 and BD3005 respectively), a 538 bp PCR product containing *nin* was cloned in pG67 (Chung and Dubnau, 1998) and cut with *SmaI* and *KpnI*. The resulting plasmid was named pRP9. The vector pG67 contains the *comG* promoter flanked by *amyE* front and back sequences, as well as genes for *Cm* and ampicillin resistance. With this vector, genes can be inserted at the *amyE* locus under control of the *comG* promoter. BD3007 and BD3005 were obtained by transforming BD2941 and BD2942 (Table 2) with pRP9, selecting for  $\text{Cm}^R$ , and screening the transformants for the loss of amylase activity.

### Construction of *nucA*-*phoA* and *nucA*-*lacZ* fusions and assay of alkaline phosphatase and $\beta$ -galactosidase activities

Fusions of *nucA* to either *phoA* and *lacZ* were generated by cloning *nucA* (amplified by PCR) in frame with the *phoA* reporter gene of plasmid pUCCMPHOA (Piazza *et al.*, 1999) or with the *lacZ* reporter gene of plasmid pJF751 (Ferrari *et al.*, 1986). A hybrid protein containing all of *nucA* fused to the 15th residue of *phoA* was constructed by designing PCR primers that placed *nucA* in frame with *phoA*. For cloning, the upstream primer contained a *BamHI* site, and the downstream primer contained a *SalI* site. Similarly, constructs containing the same fragment of *nucA* fused to the eighth codon of *lacZ* were generated with primers containing *EcoRI* and *BamHI* sites. *Bacillus subtilis* strains harbouring fusions were obtained by transformation of BD630 with these fusion plasmids using selection for  $\text{Cm}^R$ . The resulting transformants were derived from single crossover events at the *nucA* locus and carry the *nucA* fusions in single copy on the chromosome downstream of the endogenous regulatory sequences. Alkaline phosphatase activities were determined in *B. subtilis* as described (Manoil, 1991) but as modified (Piazza *et al.*, 1999). The activity units of alkaline phosphatase

are defined so as to include a term for the optical density of the culture. The  $\beta$ -galactosidase activities were determined as described previously (Piazza *et al.*, 1999).

### *B. subtilis* cell fractionation and Western blot analysis

Cells from 10 ml of culture were harvested by centrifugation at the time of maximal competence and resuspended in 0.5 ml of protoplasting buffer [28% sucrose, 10 mM Tris-hydrochloride (pH 8.0), 5 mM  $\text{MgCl}_2$ ]. Lysozyme ( $0.1 \text{ mg ml}^{-1}$ ) was added together with protease inhibitors [aprotinin ( $330 \mu\text{g ml}^{-1}$ ), leupeptin ( $165 \mu\text{g ml}^{-1}$ ) and pepstatin ( $165 \mu\text{g ml}^{-1}$ )]. The suspension was incubated for 30 min at  $37^\circ\text{C}$  and the cells were examined microscopically at intervals until protoplast formation was nearly complete. The protoplasts were sedimented and the supernatant (cell wall fraction) was precipitated in 10% TCA, washed with 80% acetone and resuspended in SDS sample buffer for electrophoresis. The protoplasts were lysed by resuspension in 1 ml of 50 mM Tris-HCl, pH 8.0, 5 mM  $\text{MgCl}_2$ , 50 mM NaCl,  $10 \mu\text{g ml}^{-1}$  DNase,  $10 \mu\text{g ml}^{-1}$  RNase, with the protease inhibitors included. This preparation was centrifuged at 55 000 r.p.m. for 20 min at  $4^\circ\text{C}$  in a TLA 100.2 rotor in a TL-100 centrifuge (Beckman). The supernatant (cytoplasmic fraction) was precipitated in 10% TCA and treated as described above, whereas the pellet (membrane fraction) was solubilized in SDS sample buffer for electrophoresis. NaOH extraction of the membrane fraction and separation into soluble and insoluble fractions were carried out as described previously (Inamine and Dubnau, 1995). Amounts of these samples from equivalent volumes of cell culture were resolved in SDS-polyacrylamide gels (Laemmli, 1970) containing 8% polyacrylamide and a 4% polyacrylamide stacking gel. The gels were equilibrated in transfer buffer (48 mM Tris-HCl, pH 9.2, 39 mM glycine, 0.05% SDS, 20% methanol) for 10 min and then electrophoretically blotted to pre-wetted nitrocellulose membranes (0.2  $\mu\text{m}$  pore size, Schleicher and Schuell) for 60 min at 12 V in a semi-dry transfer apparatus (Bio-Rad). The nitrocellulose membranes were incubated with monoclonal anti-LacZ antibody (Promega) according to the manufacturer's instructions, and the NucA-LacZ fusion protein was specifically visualized using an ECL kit (Amersham).

### Preparation of radiolabelled DNA and measurement of binding, uptake and degradation of transforming DNA

*Bacillus subtilis* high-molecular-weight [ $^3\text{H}$ ]-labelled DNA was obtained essentially as described (Dubnau and Cirigliano, 1972a). Cells grown to competence were incubated with [ $^3\text{H}$ ]-DNA ( $1.5 \times 10^5$  c.p.m.  $\mu\text{g}^{-1}$ ) added at saturating concentrations ( $0.5\text{--}1 \mu\text{g ml}^{-1}$ ) and culture samples were taken at different times. Total cell-associated and internalized DNA were determined by liquid scintillation counting after the cells were washed three times in minimal salts solution (Anagnostopoulos and Spizizen, 1961) after incubation without or with DNase ( $100 \mu\text{g ml}^{-1}$ ). DNase treatment was for 3 min at  $37^\circ\text{C}$ . Degradation of the non-transforming strand was measured as the amount of radioactivity recovered from the

supernatant after precipitation with 5% TCA and 100  $\mu\text{g ml}^{-1}$  of BSA at 0°C for 20 min, followed by centrifugation.

#### Plasmid linearization assay

*Bacillus subtilis* cells grown to competence were incubated with ethidium bromide-CsCl purified plasmid pBluescript KS<sup>-</sup> DNA (0.5  $\mu\text{g ml}^{-1}$ ) and samples (10 ml) were taken at different times and chilled on ice. After centrifugation (10 min at 4°C, 10 000 r.p.m.), cells were resuspended in 1 ml of LM buffer (0.1 M NaCl, 0.1 M EDTA, pH 6.9) and recentrifuged through a 15% sucrose column in LM to remove the unbound DNA. The pellet was washed once more in LM, resuspended in 0.4 ml of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA and incubated for 20 min at room temperature with gentle shaking in the presence of 2% Sarkosyl plus an equal volume of phenol. This treatment results in the release of DNA bound to the cell surface (Dubnau, 1976). The aqueous phase was precipitated by the addition of 50  $\mu\text{g ml}^{-1}$  tRNA and two volumes of ethanol followed by centrifugation, and the pellet was redissolved in 20  $\mu\text{l}$  of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Next, 10  $\mu\text{l}$  of this preparation was loaded on 0.8% agarose gels in 0.5 $\times$  TBE.

#### Southern hybridization

DNA was transferred to Hybond membranes (Amersham) using a VacuGene transfer apparatus (Pharmacia) according to the manufacturer's instructions. Probes were labelled using [ $\alpha$ -<sup>32</sup>P]-dCTP with a nick translation kit (Amersham).

#### Acknowledgements

We thank all the members of our laboratory and also Riccardo Manganelli for useful discussions and advice. This work was supported by NIH grant GM43756. We thank Richard Pine for a gift of pBluescript KS<sup>-</sup> DNA. We also thank an anonymous reviewer for suggesting that Nin may protect chromosomal DNA from the action of NucA.

#### References

- Albano, M., Hahn, J., and Dubnau, D. (1987) Expression of competence genes in *Bacillus subtilis*. *J Bacteriol* **169**: 3110–3117.
- Anagnostopoulos, C., and Spizizen, J. (1961) Requirements for transformation in *Bacillus subtilis*. *J Bacteriol* **81**: 741–746.
- Chung, Y.S., and Dubnau, D. (1995) ComC is required for the processing and translocation of comGC, a pilin-like competence protein of *Bacillus subtilis*. *Mol Microbiol* **15**: 543–551.
- Chung, Y.S., and Dubnau, D. (1998) All seven comG open reading frames are required for DNA binding during transformation of competent *Bacillus subtilis*. *J Bacteriol* **180**: 41–45.
- Chung, Y.S., Breidt, F., and Dubnau, D. (1998) Cell surface localization and processing of the ComG proteins, required

- for DNA binding during transformation of *Bacillus subtilis*. *Mol Microbiol* **29**: 905–913.
- Dubnau, D. (1976) Genetic transformation of *Bacillus subtilis*: A review with emphasis on the recombination mechanism. In *Microbiology 1976*. Schlessinger, D. (ed.). Washington DC: American Society for Microbiology, pp. 14–27.
- Dubnau, D. (1999) DNA uptake in bacteria. *Annu Rev Microbiol* **53**: 217–244.
- Dubnau, D., and Cirigliano, C. (1972a) Fate of transforming DNA following uptake by competent *Bacillus subtilis*. III. Formation and properties of products isolated from transformed cells which are derived entirely from donor DNA. *J Mol Biol* **64**: 9–29.
- Dubnau, D., and Cirigliano, C. (1972b) Fate of transforming DNA following uptake by competent *Bacillus subtilis*. IV. The endwise attachment and uptake of transforming DNA. *J Mol Biol* **64**: 31–46.
- Dubnau, D., and Cirigliano, C. (1974) Uptake and integration of transforming DNA in *Bacillus subtilis*. In *Mechanisms in Recombination*. Grell, R.F. (ed.). New York: Plenum Press, pp. 167–178.
- Ferrari, E., Howard, S.M.H., and Hoch, J. (1986) Effect of stage 0 sporulation mutations on subtilisin expression. *J Bacteriol* **166**: 173–179.
- Hahn, J., Albano, M., and Dubnau, D. (1987) Isolation and characterization of Tn917lac-generated competence mutants of *Bacillus subtilis*. *J Bacteriol* **169**: 3104–3109.
- Hahn, J., Inamine, G., Kozlov, Y., and Dubnau, D. (1993) Characterization of comE, a late competence operon of *Bacillus subtilis* required for the binding and uptake of transforming DNA. *Mol Microbiol* **10**: 99–111.
- Inamine, G.S., and Dubnau, D. (1995) ComEA, a *Bacillus subtilis* integral membrane protein required for genetic transformation, is needed for both DNA binding and transport. *J Bacteriol* **177**: 3045–3051.
- Lacks, S., and Neuberger, M. (1975) Membrane location of a deoxyribonuclease implicated in the genetic transformation of *Diplococcus pneumoniae*. *J Bacteriol* **124**: 1321–1329.
- Lacks, S., Greenberg, B., and Neuberger, M. (1975) Identification of a deoxyribonuclease implicated in genetic transformation of *Diplococcus pneumoniae*. *J Bacteriol* **123**: 222–232.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Londoño-Vallejo, J.A., and Dubnau, D. (1993) comF, a *Bacillus subtilis* late competence locus, encodes a protein similar to ATP-dependent RNA/DNA helicases. *Mol Microbiol* **9**: 119–131.
- Londoño-Vallejo, J.A., and Dubnau, D. (1994a) Membrane association and role in DNA uptake of the *Bacillus subtilis* PriA analog ComF1. *Mol Microbiol* **13**: 197–205.
- Londoño-Vallejo, J.A., and Dubnau, D. (1994b) Mutation of the putative nucleotide binding site of the *Bacillus subtilis* membrane protein ComFA abolishes the uptake of DNA during transformation. *J Bacteriol* **176**: 4642–4645.
- Luttinger, A., Hahn, J., and Dubnau, D. (1996) Polynucleotide phosphorylase is necessary for competence development in *Bacillus subtilis*. *Mol Microbiol* **19**: 343–356.
- Manoil, C. (1991) Analysis of membrane protein topology

- using alkaline phosphatase and  $\beta$ -galactosidase gene fusions. *Methods Cell Biol* **34**: 61–75.
- Mulder, J.A., and Venema, G. (1982a) Isolation and partial characterization of *Bacillus subtilis* mutants impaired in DNA entry. *J Bacteriol* **150**: 260–268.
- Mulder, J.A., and Venema, G. (1982b) Transformation-deficient mutants of *Bacillus subtilis* impaired in competence-specific nuclease activities. *J Bacteriol* **152**: 166–174.
- Nielsen, H., Engelbrecht, J., von Heijne, G., and Brunak, S. (1996) Defining a similarity threshold for a functional protein sequence pattern: the signal peptide cleavage site. *Proteins* **24**: 165–177.
- Piazza, F., Tortosa, P., and Dubnau, D. (1999) Mutational analysis and membrane topology of ComP, a quorum-sensing histidine kinase of *Bacillus subtilis* controlling competence development. *J Bacteriol* **181**: 4540–4548.
- Provvedi, R., and Dubnau, D. (1999) ComEA is a DNA receptor for transformation of competent *Bacillus subtilis*. *Mol Microbiol* **31**: 271–280.
- Russel, M., and Model, P. (1982) Filamentous phage pre-coat is an integral membrane protein: analysis by a new method of membrane preparation. *Cell* **28**: 177–184.
- van Sinderen, D., Luttinger, A., Kong, L., Dubnau, D., Venema, G., and Hamoen, L. (1995a) *comK* encodes the competence transcription factor, the key regulatory protein for competence development in *Bacillus subtilis*. *Mol Microbiol* **15**: 455–462.
- van Sinderen, D., Kiewiet, R., and Venema, G. (1995b) Differential expression of two closely related deoxyribonucleases, *nucA* and *nucB* in *Bacillus subtilis*. *Mol Microbiol* **15**: 213–223.
- Smith, H., Wiersma, K., Bron, S., and Venema, G. (1983) Transformation in *Bacillus subtilis*: purification and partial characterization of a membrane-bound DNA-binding protein. *J Bacteriol* **156**: 101–108.
- Smith, H., Wiersma, K., Bron, S., and Venema, G. (1984) Transformation in *Bacillus subtilis*: a 75,000-dalton protein complex is involved in binding and entry of donor DNA. *J Bacteriol* **157**: 733–738.
- Smith, H., Wiersma, K., Venema, G., and Bron, S. (1985) Transformation in *Bacillus subtilis*: Further characterization of a 75,000-Dalton protein complex involved in binding and entry of donor DNA. *J Bacteriol* **164**: 201–206.
- Steinmetz, M., and Richter, R. (1994) Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through *in vivo* recombination. *Gene* **142**: 79–83.
- Vosman, B., Kuiken, G., and Venema, G. (1988) Transformation in *Bacillus subtilis*: involvement of the 17-kilodalton DNA-entry nuclease and the competence-specific 18-kilodalton protein. *J Bacteriol* **170**: 3703–3710.