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The *Serratia marcescens* NucE Protein Functions as a Holin in *Escherichia coli*

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The recently discovered *nucC* locus of *Serratia marcescens* encodes the cryptic prophage genes *nucE*, *nucD*, and *nucC*. NucC is required for expression of the *S. marcescens* nuclease and functions as a transcriptional activator of the nuclease gene, *nucA*. NucE and NucD are dispensable for nuclease expression but were proposed to allow for secretion of the nuclease by *Escherichia coli*. Here, we show (i) that the NucE protein is membrane bound, (ii) that it can complement the λ S holin, (iii) that it can be triggered by potassium cyanide, (iv) that it is detrimental to cell viability, and (v) that the concomitant expression of *nucE* and *nucD* results in cell lysis. Apparently NucE and NucD function as a holin and an endolysin, respectively. This suggests that their roles in nuclease secretion by *E. coli* are indirect, possibly through directed cell lysis.

By using transposon mutagenesis, *Serratia marcescens* mutations causing defects in nuclease production have been mapped to the *nucC* locus (7). The *nucC* locus comprises three genes: *nucC*, encoding a transcriptional activator with homology to the P2 Ogr protein (8), as well as *nucE* and *nucD*, encoding a putative holin and a putative endolysin, respectively. The presence of genes with similarities to a phage transcriptional activator and lysis genes suggested that the *nucC* locus may be part of a cryptic prophage. In *Escherichia coli* NucE, and possibly NucD, were shown to allow for extracellular secretion of the nuclease. *nucE* encodes an 89-amino-acid-long protein with characteristic traits of holins. It has highly charged N and C termini and two putative transmembrane domains separated by a β turn region (Fig. 1). The protein encoded by the *nucD* gene has high sequence homology to the V-type lysozymes, encompassing eight highly conserved residues (2). Holins represent a class of phage lysis proteins which induce a lesion in the cytoplasmic membrane through which the phage endolysin is released to its murein substrate. As a consequence, cell lysis ensues immediately and progeny phages are released (for a review, see references 12 and 13). In this note, we show that the *nucE* gene product functions as a holin in *E. coli* and that it functionally complements the S holin of bacteriophage λ.

Construction of plasmids. To produce a DNA fragment containing the *S. marcescens* genes *nucE* and *nucD*, the oligonucleotides L-8 (5′-AAAAA AAAGA ATTCC ATACA GGCCA GGAGA TGATG G-3′) and A-9 (5′-AAAAA AAAAG TACTC ATCCT TTGGC GAGCG AACTC-3′) were used as primers for PCR amplification. Plasmid pZ150Bam3.3 (Table 1) served as the template for the PCR performed with *Pfu* DNA polymerase. The primers L-8 and A-9 hybridize to the upstream and downstream sequences, respectively, of the *nucE* and *-D* reading frames. They introduced an *Eco*RI and *Sca*I site 5′ and 3′, respectively, of *nucE* and *-D*. The PCR product was digested with *Eco*RI and *Sca*I, and the resulting 845-bp fragment was cloned into the *Eco*RI-*Hin*cII sites of plasmid pBluescript II KS(+), giving rise to plasmid pKSLA (Table 1). Plasmid pKSLA was subsequently digested with *Hin*cII and *Apa*I and religated. This manipulation removed the *nucD* gene and resulted in plasmid pKSE, which carries only the *nucE* gene.

Complementation of a λ *Sam* mutant phage by NucE. Henrich et al. (6) have recently shown that the *Lactobacillus gasseri* bacteriophage φadh holin complements the *Sam*100 mutation of the λ cloning vector gt11 in a nonsuppressing *E. coli* strain. To gain a preliminary indication of whether NucE functions as a holin, we used the same experimental approach. An *Eco*RI linker was first ligated to the *Eco*RI-*Hin*cII fragment of plasmid pKSLA. The fragment, containing the entire *nucE* gene and part of the *nucD* gene, was then digested with *Eco*RI and ligated to λgt11 arms. The λ DNA was packaged by using the Gigapack II packing system from Stratagene cloning systems. *E. coli* Y1089r$^-$ (Table 1) was infected with the packing mixture. It was anticipated that plaque formation would depend

TABLE 1. Strains, phage, and plasmids used in this study

Strain, phage, or plasmid	Genotype or relevant features	Source or reference
Strains		
KT842	p_{lac} *UV5*-T7 gene *1-kan*::′*lacZ* [F′ *proAB lacI*^{q1}*ZΔM15* Tn*10* (Tet^r)]	11
Y1089r$^-$	Δ(*lac*)*U169* Δ(*lon*) *araD139 strA mcrB hflA150*::Tn*10* (Tet^r) [pMC9]	Stratagene
Y1090r$^-$	Δ(*lac*)*U169* Δ(*lon*) *araD139 strA supF mcrA mcrB hsdR trpC22*::Tn*10* (Tet^r) [pMC9]	Stratagene
Phage λgt11	*cI*857 *Sam*100 p_{lac5}	Stratagene
Plasmids		
pKSII(+)	Ap^r p_{lac} *lacZ*′ T7p T3p	Stratagene
pKSLA	845-bp PCR product containing *nucED* in pKSII(+)	This work
pKSE	Deletion of the *Hin*cII-*Apa*I *nucD* fragment from pKSLA (*nucE*+)	This work
pZ150Bam3.3	*nucEDC* on 3.3-kb *Bam*HI fragment in pZ150	7

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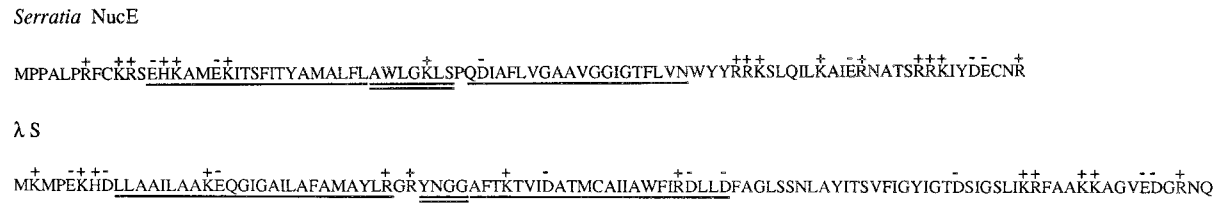

FIG. 1. Structural similarity of NucE to phage λ holin S. +, −, charged residues; —, potential transmembrane domains; ═, potential β turns.

on complementation of the defective λ *Sam*100 allele by *nucE*. From several white plaques, recombinant λ phages were obtained. The insertion of the *nucE* fragment was verified in all selected plaques by PCR amplification (data not shown). The plating efficiencies of several individual recombinant lambda phages tested were generally two orders of magnitude lower on the nonsuppressing strain, Y1089r$^-$, than on the *supF* reference strain, Y1090r$^-$. This indicated that the plaque-forming ability of the recombinant lambda phages did not result from a reversion of the *Sam*100 allele present in the cloning vector and that it is attributable to the NucE function. To unambiguously demonstrate the presence of the *Sam*100 mutation in the recombinant phages, the *S* gene sequence was verified from a recombinant phage. Since the location of the *Sam*100 mutation was unknown, the complete *S* gene sequence of both strands was determined by automated sequencing. The *Sam*100 mutation was found to reside in codon 10 of the *S* gene. This result indicated that NucE can complement the *S* defect of λgt11, albeit with a somewhat reduced efficiency as indicated by the reduced efficiency of plating on the nonsuppressing strain, Y1089r$^-$.

NucE is membrane bound. To determine the subcellular localization of NucE, plasmids pKSE and pKSII(+) were introduced into strain KT842 (Table 1), bearing a chromosomal copy of the T7 RNA polymerase gene *1*. Expression of T7 gene *1* was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 5 mM. Labelling with [^{35}S]methionine, as well as preparation of the cytoplasmic and membrane fractions of strain KT842 harboring plasmid pKSE or pKSII(+), was performed as previously described (10). As shown in Fig. 2, lane 1, the NucE protein with an apparent M_r of approximately 10,000 was exclusively detected in the membrane fraction. NucE was not visible in the cytoplasmic fraction of KT842(pKSE) (Fig. 2, lane 2) nor in either the membrane (Fig. 2, lane 3) or cytoplasmic fraction (Fig. 2, lane 4) of the control strain, KT842[pKSII(+)].

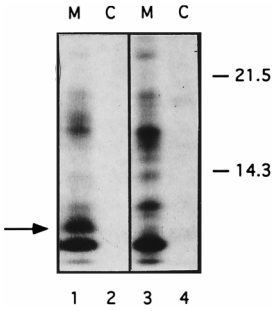

FIG. 2. NucE is membrane bound. *E. coli* KT842(pKSE) and KT842[pKSII(+)] were grown in minimal media and labeled with [^{35}S]methionine, and the membrane (M) and cytosolic (C) fractions were isolated, as previously described (10). The samples were analyzed on a sodium dodecyl sulfate–17.5% polyacrylamide gel. Lanes 1 and 2, membrane and cytoplasmic fractions of KT842(pKSE); lanes 3 and 4, membrane and cytoplasmic fractions of KT842 [pKSII(+)]. The position of NucE is indicated by the arrow. The positions of the marker proteins, soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa), are indicated at the right.

NucE is lethal for *E. coli*. To test whether NucE induces a lethal lesion in the cytoplasmic membrane, one hallmark of holin proteins, the cell viability was measured at 30-min intervals upon induction of the *nucE* gene in strain KT842(pKSE). As shown in Table 2, in contrast to the control strain, KT842 [pKSII(+)], the viability of strain KT842(pKSE) decreased very rapidly. This result showed that NucE behaves analogously to λ *S* clones (5). Next, we tested whether concomitant expression of NucE and NucD results in host cell lysis. As shown in Fig. 3, simultaneous expression of the NucE and NucD genes resulted in lysis of strain KT842(pKSLA) 52 min after induction. Chloroform, which permeabilizes the cytoplasmic membrane, resulted in premature lysis when it was added to strain KT842(pKSLA) 6 min after induction of the NucE and -D genes (Fig. 3), suggesting that the NucD protein functions as an endolysin and that NucE is required for transit of the endolysin to the periplasm.

Most, if not all, holin proteins (1, 12, 13) are unique in that they are the only examples of integral inner membrane proteins that require dissipation of the membrane potential for functional assembly, i.e., formation of the membrane lesion. It has recently been suggested that holins exist in the cytoplasmic membrane in a chronic state, in which the integrity of the membrane is preserved, and in an acute state, in which the holin-dependent lesion is formed (1). It is not known whether the chronic and acute states differ at the conformational level or the oligomerization state or both. The conversion of holins from the chronic to the acute state can apparently be triggered prematurely by the addition of energy poisons (1). Figure 3 shows that addition of KCN 6 min after induction of both *nucE* and *nucD* gene expression in strain KT842(pKSLA) resulted in immediate lysis, which was found to be strictly dependent on the presence of the *nucE* gene (not shown). Apparently, NucE shares this typical trait with other well-defined holins.

TABLE 2. Loss of viability after induction of *nucE* transcription

Time[a] (min)	pKSII(+) (% CFU)[b]	pKSE (% CFU)[b]	% pKSE/ % pKSII(+)
0	100	100	1
30	692	13	1.9×10^{-2}
60	653	0.4	6.1×10^{-4}
90	477	0.3	6.3×10^{-4}
120	846	0.2	2.4×10^{-4}

[a] Samples were withdrawn at times indicated.

[b] *E. coli* KT842[pKSII(+)] and KT842(pKSE) were grown in LB medium (9) at 28°C, supplemented with 0.2% glucose to an A_{600} of 0.2, and induced with IPTG (final concentration, 5 mM). Duplicates of the withdrawn samples were plated out in serial dilutions on LB plates containing 50 μg of kanamycin, 120 μg of ampicillin, and 10 μg of tetracycline per ml. The colony-forming units at time zero were taken as 100%, and the values were calculated relative to the number of colony-forming units at time zero.

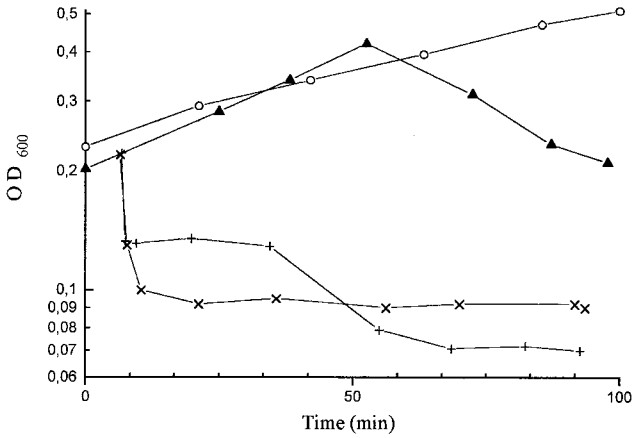

FIG. 3. NucE and NucD function as a holin and an endolysin, respectively. *E. coli* KT842(pKSLA) was cultured in Luria-Bertani (LB) medium (9) at 28°C supplemented with 0.2% glucose in the absence (○) or presence (▲, ×, +) of IPTG. IPTG (final concentration, 5 mM) was added at time zero. Six minutes after the addition of IPTG, aliquots of the culture were withdrawn and either potassium cyanide (×; final concentration, 10 mM) or chloroform (+; final concentration, 0.8% [vol/vol]) was added. OD_{600}, optical density at 600 nm.

Taken together, the data presented in this note strongly suggest that NucE functions as a holin and NucD functions as an endolysin. Ferrer et al. (3) have shown that synthesis of bacteriocin 28b upon mitomycin induction by a different *S. marcescens* strain is mediated by the transcriptional activator RegC, which is analogous to the NucC protein. It remains to be seen whether the NucED proteins (RegAB in the Ferrer et al. system) play a role in the release of bacteriocin 28b by cell lysis after mitomycin C induction, which activates expression of the *nucEDC* (*regCBA*) operon (3, 7). Preliminary data from *nucED* deletions in *S. marcescens* suggest that nuclease is still secreted in their absence.

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