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Global regulator H-NS and lipoprotein NlpI influence production of extracellular DNA in *Escherichia coli*

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ABSTRACT

Extracellular DNA (eDNA) is a structural component of the polymeric matrix of biofilms from different species. Different mechanisms for DNA release have been proposed including lysis of cells, lysis of DNA-containing vesicles, and DNA secretion. Here, a genome-wide screen of 3985 non-lethal mutations was performed to identify genes whose deletion alters eDNA release in *Escherichia coli*. Deleting *nlpI*, *yfeC*, and *rna* increased eDNA from planktonic cultures while deleting *hns* and *rfaD* decreased eDNA production. The lipoprotein NlpI negatively affects eDNA release since the overexpression of *nlpI* decreases eDNA 16 fold while deleting *nlpI* increases eDNA threefold. The global regulator H-NS is required for eDNA production since DNA was not detected for the *hns* mutant and production of H-NS restored eDNA production to wild-type levels. Therefore our results suggest that secretion may play a role in eDNA release in *E. coli* since the effect of the *hns* deletion on cell lysis (slight decrease) and membrane vesicles (threefold increase) does not account for the reduction in eDNA.

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1. Introduction

Bacteria accumulate at interfaces forming biofilms, communities of cells embedded in a self-produced polymeric matrix. The matrix constitutes about 90% of the mass of the biofilm and mainly consists of extracellular polysaccharides, proteins, lipids, and nucleic acids [1]. The extracellular DNA (eDNA) component of the biofilm matrix has been found in many Gram positive [2–5] and Gram negative bacteria [6–8] and serves many roles in different bacteria; eDNA is required for initial attachment to a surface [2,5,9], has a structural role connecting the cells in the biofilm [2,7,10,11], works as a nutrient source [12,13], contributes to cation gradients, induces antibiotic resistance, and promotes its own release via cell lysis by destabilizing membranes through cation chelation [14]. eDNA also facilitates horizontal gene transfer and DNA uptake [15].

eDNA also works as an interconnecting material for planktonic cells for *Pseudomonas aeruginosa* where microscopic observation and DNase I treatment indicate that planktonic cells are connected by eDNA-forming clumps [11]. Another example is the marine photosynthetic bacterium *Rhodovulum sulfidophilum* which forms aggregated communities of cells called flocs [16] joined by extracellular DNA and RNA [17].

The origin of eDNA is not clear since some reports indicate that eDNA is similar to genomic DNA (gDNA) [9,11,16] but other studies revealed, by comparing eDNA and gDNA through random amplification, that they are different [10,17]. The most common mechanism of eDNA release is cell lysis [9,11,15,18]. However, it has been proposed that membrane vesicles (MVs) released from the outer membrane also participate in eDNA production [6] since when MVs are opened, eDNA and enzymes that promote lysis are liberated [19]. Some bacteria produce eDNA by direct secretion from intact cells such as *Neisseria gonorrhoeae* that produces eDNA via type IV secretion system [20].

eDNA release has been related to quorum-sensing in *Streptococcus pneumoniae* via the competence-stimulating peptide (CSP) [15] and in *P. aeruginosa* via acylhomoserine lactones (AHLs) and PQS signaling [11]. We also have reported that eDNA levels are inversely related to c-di-GMP in *P. aeruginosa* [21] as regulated by tyrosine phosphorylation regulator TpbA [22].

Here, we sought to identify the genes controlling the release of eDNA in *E. coli* [23] in order to understand better the nature of its release from this strain; to date the mechanism of DNA release in this best-studied strain has not been addressed. We screened the entire Keio collection of 3985 *E. coli* K-12 BW25113 single gene knock-out mutants for eDNA using a fluorescence dye to stain the DNA present in the supernatant of cultures grown quiescently in minimal media in microtiter plates. The mutations altering eDNA production are related to general cellular processes such as

Abbreviations: eDNA, extracellular DNA; MVs, membrane vesicles; gDNA, genomic DNA; CSP, competence-stimulating peptide; AHLs, acylhomoserine lactones; dsDNA, double stranded DNA; LB, Luria-Bertani; qPCR, quantitative polymerase chain reaction; LPS, lipopolysaccharide.

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DNA replication, transcription, translation, nutrient transport and metabolism, and cell envelope. Specifically, the *nlpI*, *yfeC*, and *rna* mutants increased eDNA production and the *hns* and *rfaD* mutants decreased eDNA production. The role of cell lysis and MVs on eDNA with the *nlpI* and *hns* mutants was also investigated; these results suggest DNA is secreted by a process controlled by H-NS.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

The *E. coli* strains and plasmids used in this study are listed in Table 1. We used the 3985 *E. coli* K-12 BW25113 single gene knock-out mutants from the Keio collection [24] for the eDNA screening and the ASKA library [25] for overexpression of specific genes. Cultures were made in Luria-Bertani (LB) [26]. Kanamycin (50 μg/mL) was used for pre-culturing the knock-out mutants, carbenicillin (100 μg/mL) was used for pLP170, and chloramphenicol (30 μg/mL) was used for selecting plasmid pCA24N and its derivatives. All experiments were conducted at 37 °C.

2.2. eDNA screening

The mutants from the Keio collection were transferred from glycerol stocks, using a 96 pin replicator (Boekel Scientific, Feasterville, PA), to 96-well polystyrene plates (Corning, Lowell, MA) containing 300 μL of AB medium [27] supplemented with 0.2% glucose and 0.4% casamino acids and were incubated for 24 h without shaking. AB medium [11] was used for the screening since LB medium interfered with the fluorescence dye used for detecting eDNA. Cell density was measured at 620 nm with a Sunrise microplate reader (Tecan, Salzburg, Austria), and the 96-well plates were centrifuged at 4150 rpm for 10 min using an AccuSpin 3R centrifuge (Fisher Scientific Co, Pittsburgh, PA). The amount of DNA in 100 μL of supernatant was determined with Quant-iT PicoGreen dsDNA kit (Molecular Probes, Eugene, OR) using a Spectra Max Germini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 480 nm and emission wavelength of 520 nm. The amount of DNA was normalized by the cell density, and the mutants that significantly altered eDNA were screened again against the wild-type BW25113 using at least three independent colonies of each strain.

2.3. Quantitative polymerase chain reaction (qPCR)

eDNA was purified as described previously [21] from cells cultured for 24 h in LB with shaking at 250 rpm starting from an initial turbidity at 600 nm of 0.05. The culture (1 mL) was centrifuged at 13 krpm for 10 min, and the supernatant was used for eDNA purification using phenol/chloroform/isoamyl alcohol (25:24:1) extraction and sodium acetate and isopropanol precipitation. To normalize the eDNA by the total amount of DNA in the cells and in the supernatant, one millilitre of culture was sonicated for 45 s at 10 W (60 Sonic Dismembrator, Fisher Scientific Co, Pittsburgh, PA) and centrifuged at 13 krpm for 10 min; the supernatant was used for total DNA purification. The purified eDNA and total DNA from at least two independent cultures of each strain was quantified by qPCR using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) and the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) with primers for the reference gene *purA* (purA-f 5′-GGGCCTGCTTATGAAGATAAAGT-3′ and purA-r 5′-CAACCACCATAGAAGTCAGGT-3′).

2.4. Cell lysis assay

BW25113 and the *hns* and *nlpI* mutants expressing *lacZ* from pLP170 were cultured into 25 mL of LB medium starting from a cell density of 0.05 at 600 nm for 24 h, 250 rpm. The β-galactosidase activity of the culture supernatants was normalized by the total β-galactosidase activity of the sonicated cultures and used to evaluate cell lysis as described previously [28].

2.5. Membrane vesicles

MVs were purified as described previously [29], with some modifications. BW25113, *nlpI*, and *hns* cultures in LB with an initial turbidity at 600 nm of 0.03 were grown for 14 h then centrifuged at 6000 *g* for 10 min at 4 °C. The supernatants were filtered through a 0.22 μm vacuum filter (Millipore Co., Billerica, MA) and concentrated by ultrafiltration using a 100 kDa cut-off Diaflo membrane (Amicon Co., Lexington, MA) in a stirred ultrafiltration cell (model 8200, Amicon Co., Lexington, MA). The concentrated supernatants were ultracentrifuged at 30 krpm for 1 h at 4 °C in a SW41 Ti rotor (154,100 g) using a Beckman L8-M ultracentrifuge (Beckman Coulter Inc., Brea, CA); the supernatants were decanted and the precipitated membrane vesicles were resuspended with 50 mM HEPES

Table 1
Escherichia coli strains and plasmids used in this study.

Strains and plasmids	Genotype/relevant characteristics[a]	Source
Strains		
BW25113	F^- *Δ(araD-araB)567 ΔlacZ4787*(::rrnB-3) λ^- *rph-1* *Δ(rhaD-rhaB)568 hsdR514*; parental strain for the Keio collection.	Yale Coli Genetic Stock Center
BW25113 *hns*	BW25113 *Δhns746::kan* Km^R	[24]
BW25113 *nlpI*	BW25113 *ΔnlpI775::kan* Km^R	[24]
BW25113 *rfaD*	BW25113 *ΔrfaD731::kan* Km^R	[24]
BW25113 *rna*	BW25113 *Δrna749::kan* Km^R	[24]
BW25113 *yfeC*	BW25113 *ΔyfeC732::kan* Km^R	[24]
BW25113 *hha*	BW25113 *Δhha745::kan* Km^R	[24]
BW25113 *hha hns*	BW25113 *Δhha845 Δhns746::kan* Km^R	[48]
Plasmids		
pCA24N	$lacI^q$, Cm^R	[25]
pCA24N-*hns*	pCA24N $P_{T5\text{-}lac}$::*hns* Cm^R	[25]
pCA24N-*nlpI*	pCA24N $P_{T5\text{-}lac}$::*nlpI* Cm^R	[25]
pLP170	Promoterless *lacZ* fusion vector Cb^R	[49]

[a] Km^R, Cm^R, Cb^R are kanamycin, chloramphenicol, and carbenicillin resistance, respectively.

pH 6.8 buffer. The amount of MVs was determined using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

3. Results

3.1. Screening of the genes involved in eDNA production

To identify genes involved in *E. coli* eDNA production, we screened 3985 nonessential gene knock-out mutants of the Keio Collection [24]. Since the mutants were grown quiescently in microtiter plates, the eDNA detected in the screening was produced from both biofilm and planktonic cells. The screening was performed based on the fluorescence of Quant-iT PicoGreen reagent upon binding to double stranded DNA (dsDNA); the sensitivity with the conditions used for the assay was 0.004 ng dsDNA/μL. After two rounds of screening, four mutants that increase eDNA and 31 mutants that decrease eDNA more than 2.5 fold were identified (Table 2). These genes encode proteins mainly located in the cytoplasm that are related to different cellular processes including the synthesis of components of the cell envelope such as lipopolysaccharide (LPS).

Fifteen mutants indentified in the initial screen which had the biggest impact on eDNA within various functional groups were further verified via qPCR with eDNA samples purified from planktonic cells cultured in LB: *rna*, *hns*, *pnp*, *groL*, *cyaA*, *aspC*, *moaE*, *menD*, *pstA*, *rfaD*, *rfaG*, *ybgF*, *nlpI*, *yfeC*, and *yieL*. Of these 15, the *nlpI*, *yfeC*, and *rna* mutants increased eDNA, and the *rfaD* mutant decreased eDNA as expected based on the initial screen (Fig. 1A). There was no amplification via qPCR for the eDNA samples of the *hns* mutant (even after a 50-fold concentration); hence, the *hns* deletion abolishes the formation of eDNA.

Hha is a global regulator [30] with nonspecific DNA binding [31] that alters the production of multiple proteins [32] and which forms a complex with H-NS that binds DNA [31]. To evaluate if the H-NS regulation of eDNA occurs through its interaction with Hha, we evaluated via qPCR the eDNA produced by the *hha* mutant and *hha hns* double mutant. The *hha* mutant produces the same

Table 2
Escherichia coli BW25113 genes whose mutations altered eDNA as detected by Quant-iT PicoGreen. The BW25113 value corresponds to the average of 31 independent colonies and for each mutant at least three colonies were assayed. For all listed mutants, differences in eDNA compared to the wild-type are significant based on a Student's *T* test ($p < 0.05$). Locations are from [50] and function are from [51,52].

Strain	OD_{620}	ng DNA μL^{-1} $OD_{620}{}^{-1}$	Location	Fold	Function
BW25113	1.1 ± 0.2	0.53 ± 0.07	–	1	–
Replication, recombination and repair					
priA	0.62 ± 0.03	0.17 ± 0.01	C	−2.9	PriA participates in DNA replication
Transcription and translation					
rna	0.9 ± 0.2	2.2 ± 0.6	P	4.4	RNase I, cleaves phosphodiester bonds in RNA
hns	0.67 ± 0.05	0.17 ± 0.02	C	−2.9	DNA-binding global regulator H-NS
pnp	1.1 ± 0.1	0.16 ± 0.01	C	−3.1	PNPase, involved in general mRNA degradation
Posttranslational modification, protein turnover, chaperones					
groL	1.2 ± 0.3	0.15 ± 0.01	C	−3.3	Chaperone Hsp60
sspA	1.1 ± 0.2	0.16 ± 0.01	C	−3.1	Protein essential for cell survival under acid-stress
Metabolism					
cyaA	0.99 ± 0.07	0.10 ± 0.01	C	−5.0	Adenylate cyclase CyaA catalyzes the synthesis of cyclic AMP
aspC	0.76 ± 0.02	0.11 ± 0.01	C	−4.5	Aspartate aminotransferase
gmhB	0.85 ± 0.08	0.13 ± 0.02	C	−3.8	D,D-heptose 1,7-bisphosphate phosphatase
btuB	1.0 ± 0.3	0.15 ± 0.06	OM	−3.3	Receptor for transport of vitamin B12, E colicins, and phages BF23 and C1
moaA	0.7 ± 0.04	0.15 ± 0.03	C	−3.3	Protein that participates in the synthesis of molybdopterin guanine dinucleotide
moaC	0.59 ± 0.04	0.19 ± 0.03	C	−2.6	Protein that participates in the MPT biosynthesis
moaE	0.8 ± 0.3	0.13 ± 0.03	C	−3.8	MPT synthase
mog	0.82 ± 0.07	0.21 ± 0.01	C	−2.8	Protein that participates in the MPT biosynthesis
menD	1.16 ± 0.05	0.14 ± 0.02	C	−3.6	Protein that participates in menaquinone biosynthesis
menE	1.14 ± 0.08	0.11 ± 0.01	C	−4.5	Protein that participates in menaquinone biosynthesis
nudB	1.1 ± 0.2	0.20 ± 0.04	C	−2.5	Protein that participates in the early steps in folate synthesis
Inorganic ion transport					
pstA	1.7 ± 0.07	0.12 ± 0.01	IM	−4.2	Part of the ATP-dependent phosphate uptake system PstABCS
pstS	1.0 ± 0.3	0.13 ± 0.04	P	−3.8	Part of the ATP-dependent phosphate uptake system PstABCS
phoU	1.1 ± 0.3	0.15 ± 0.04	C	−3.3	Negative regulator of Pho regulon (phosphate transport system)
modC	0.6 ± 0.2	0.18 ± 0.01	C	−2.8	ATP-binding component of the molybdate ABC transporter
Cell envelope					
lpcA	0.65 ± 0.08	0.19 ± 0.02	C	−2.6	Catalyzes the first step in the synthesis of core lipopolysaccharide (LPS)
rfaD	0.63 ± 0.02	0.16 ± 0.03	C	−3.1	Involved in the synthesis of the precursor of core LPS
rfaE	0.61 ± 0.05	0.16 ± 0.01	C	−3.1	Involved in the synthesis of the precursor of core LPS
rfaF	0.75 ± 0.07	0.20 ± 0.02	C	−2.5	LPS heptosyltransferase II
rfaG	0.7 ± 0.2	0.19 ± 0.04	C	−2.6	Glucosyltransferase I involved in LPS core biosynthesis
nlpD	1.0 ± 0.1	0.17 ± 0.02	OM	−2.9	Protein related to cell division
tolC	0.6 ± 0.2	2.0 ± 0.4	OM	4.0	Porin component of several multi-drug efflux systems
ybgF	0.86 ± 0.03	0.18 ± 0.02	P	−2.8	Part of the Tol-Pal contributing to maintain cell envelope integrity
yfgA	0.7 ± 0.1	0.18 ± 0.04	IM	−2.8	Protein responsible for maintaining the rod shape of the *E. coli* cell
Function unknown					
nlpI	0.7 ± 0.2	2.1 ± 0.1	OM	4.2	Lipoprotein related to osmotic sensitivity, filamentation, and virulence
yfeC	0.93 ± 0.03	1.4 ± 0.3	C	2.8	Predicted DNA-binding transcriptional regulator
yieL	0.70 ± 0.02	0.15 ± 0.01	P	−3.3	Predicted xylanase
yhbP	0.63 ± 0.03	0.18 ± 0.01	C	−2.8	Function unknown.
yjiP	1.10 ± 0.07	0.2 ± 0.1	C	−2.6	Predicted transposase involved in biofilm formation

C, cytoplasm; IM, inner membrane; P, periplasm; OM, outer membrane.

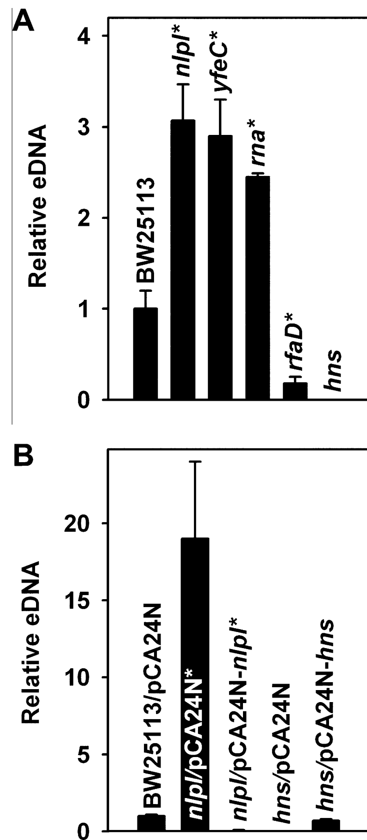

Fig. 1. eDNA quantified by qPCR. The values are the average of at least two independent cultures assayed in duplicate, the error bars correspond to the standard deviation, and an asterisk indicates p-values <0.05 using Student's T test. (A) Knock-outs mutants that altered eDNA production. Cells were grown in LB for 24 h, 250 rpm at 37 °C. (B) Complementation of *hns* and *nlpI* eDNA. Cells were grown in LB for 24 h, 250 rpm at 37 °C with 21.5 h of induction with 0.1 mM IPTG.

amount of eDNA as wild-type BW25113, and the eDNA of the *hha hns* mutant was not detected; therefore, H-NS regulation of eDNA is not related to Hha.

The *aspC*, *ybgF*, *moaE*, *menD*, *pstA*, *cyaA*, *pnp*, and *yieL* mutants did not have statistically significant differences in their eDNA compared to the wild-type strain as assayed by qPCR. The *rfaG* (2.4 fold) and *groL* (2.9 fold) mutations increased eDNA via qPCR but decreased eDNA based on the initial screening with Quant-iT PicoGreen. These discrepancies may be due to differences in the growth conditions since the initial screen was performed with cells grown quiescently in AB minimal media supplemented with glucose and casamino acids in microtiter plates, but the cells for the qPCR screen were grown in LB media in flasks with shaking.

3.2. Complementation of nlpI and hns eDNA

The mutants with the highest impact on eDNA were *nlpI* and *hns*. To confirm that NlpI and H-NS regulate eDNA production, plasmids pCA24N-*hns* and pCA24N-*nlpI* were used to overexpress *hns* and *nlpI* (Fig. 1B). As expected, eDNA was not produced by BW25113 *hns*/pCA24N, and overexpressing *hns* in BW25113 *hns*/pCA24N-*hns* restored eDNA to 70% of the wild-type BW25113/pCA24N. Similarly, as expected, the *nlpI* mutation in BW25113 *nlpI*/pCA24N increased eDNA 19 fold while overexpressing *nlpI* in BW25113 *nlpI*/pCA24N-*nlpI* decreased eDNA 16 fold (Fig. 1B). Hence, our results indicate that H-NS enhances eDNA production and that NlpI negatively controls eDNA in *E. coli*.

3.3. Cell lysis assay

Since β-galactosidase is a cytoplasmic enzyme, its activity in culture supernatants has been used previously to determine if eDNA production occurs via lysis of a subpopulation of the culture [2,11,15,18]. Therefore, plasmid pLP170 harboring *lacZ* was electroporated into BW25113 containing the *hns* or *nlpI* mutations to evaluate the β-galactosidase in the culture supernatants normalized by the β-galactosidase activity of cell lysates; *lacZ* is inactivated in wild-type BW25113. The *nlpI* mutant increased cell lysis 6.4 ± 0.9 fold which is similar to its increase in eDNA (3.1 ± 0.4 fold). However, cell lysis does not explain the decrease in eDNA in the *hns* mutant since the deletion of *hns* abolished *E. coli* eDNA production but decreased cell lysis by 1.8 ± 0.6 fold. These results suggest that cell lysis contributes to eDNA release in *E. coli*; however, another mechanism may also be present.

3.4. Membrane vesicles

To investigate whether MVs were altered by the *nlpI* and *hns* mutations, we purified MVs from supernatants of BW25113 and the *nlpI* and *hns* mutants cultures made in LB medium. The *nlpI* mutant has 107-fold more vesicles (cf., threefold more eDNA) and the *hns* mutant has threefold more vesicles (cf., no eDNA) than the wild-type BW25113. These results for MVs agree with the values reported previously for the *nlpI* [33] and *hns* [34] mutants. Therefore, eDNA production via MVs is not the main mechanism of eDNA production in *E. coli* since the changes in MVs do not match the changes in eDNA for these two mutants.

4. Discussion

Our results show that *E. coli* releases eDNA during static growth, where there are planktonic and sessile cells. Furthermore, we identified 35 proteins with a greater than 2.5-fold difference in eDNA production and characterized the *nlpI* and *hns* mutations more fully. Mutations in *yfeC*, *rna*, and *nlpI* increased eDNA. YfeC is an uncharacterized protein that has a helix-turn-helix domain [35]; hence, it probably is a negative transcriptional regulator of genes encoding proteins related to eDNA. *rna* encodes RNase I; hence, the increase in eDNA by the *rna* deletion may be related to the reduced degradation of DNA that occurs in the *rna* mutant [36]. Since RNase I is a periplasmic protein, the increase in eDNA by the *rna* deletion suggests that DNA is present in the periplasm which agrees with eDNA release via secretion.

The largest increase in eDNA was obtained with the *nlpI* mutant (Fig. 1A). NlpI is an outer membrane lipoprotein that probably participates in cell division [37] and is related to bacterial virulence in pathogenic *E. coli* strains by promoting adhesion to intestinal epithelial cells [38] and human brain microvascular endothelial cells [39]. The *nlpI* mutant shows elongation at 42 °C at low osmolarity [37] and produces more than 100-fold more membrane vesicles [33]. Cells overexpressing *nlpI* have a prolate ellipsoidal shape and have some cells joined by partial constrictions which suggest that cell division is altered due to defects in chain elongation and the formation of the septal ring [37]. Hence, deletion of *nlpI* probably leads to more eDNA that may decorate the exterior of the cell and render it less able to bind epithelial and endothelial cells.

Mutations in *hns* and *rfaD* decreased eDNA. RfaD is an enzyme that participates in the synthesis of a precursor of LPS. The *rfaD* mutant forms mini-cells which indicate cell division defects, has a mucoid phenotype, has resistance to λ phage, and cannot grow at temperatures higher than 42 °C or in media containing bile salts [40].

For the *hns* mutant, eDNA production was abolished since eDNA was not detected by qPCR. H-NS is an abundant protein

(approximately 20,000 copies per cell) [41] that binds to DNA and condenses the nucleoid [42]. H-NS functions as a transcriptional global regulator controlling genes encoding proteins related to the cell envelope and adaptation to environmental conditions [43] including 69% of temperature regulated genes [44]. The *hns* mutant forms threefold more membrane vesicles [34] and has altered chromosome partitioning and replication [45]. Since the reduction in cell lysis by the H-NS mutant is not comparable to the reduction in eDNA production, *E. coli* should have another mechanism other than lysis for eDNA production. Similarly, the production of eDNA via membrane vesicles may not be the main mechanism of eDNA production in *E. coli* since the *hns* deletion increases vesiculation but decreases eDNA. Hence, it is possible that *E. coli* produces eDNA via direct secretion from living cells. Therefore, although speculative, our data suggest that H-NS regulates eDNA secretion in *E. coli* in a manner that is not dependent on Hha.

Given that *E. coli* is a Gram negative bacterium, to be secreted, DNA should go through the inner membrane, the cell wall, and the outer membrane. This transport of DNA may also occur through the points where the inner and outer membranes are joined to each other through the cell wall [46]. The inner and outer membranes are involved in DNA replication, and the outer membrane fractions contain newly replicated DNA. During cell division, on each side of the septum, two rings are formed where the inner and outer membranes are fussed. Since both *nlpI* and *hns* mutants have altered cell division and have the biggest effect on eDNA release, secretion in *E. coli* may be related to DNA replication and cell division. An eDNA secretion mechanism related to DNA replication occurs in the Gram positive *Bacillus subtilis*. During spore germination, *B. subtillis* releases eDNA following replication, and the rate of DNA synthesis is similar to the rate of DNA release [47].

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