EliA is required for inducing the stearyl alcohol-mediated expression of secretory proteins and production of polyester in *Ralstonia* sp. NT80

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Addition of stearyl alcohol to the culture medium of *Ralstonia* sp. NT80 induced expression of a significant amount of secretory lipase. Comparative proteomic analysis of extracellular proteins from NT80 cells grown in the presence or absence of stearyl alcohol revealed that stearyl alcohol induced expression of several secretory proteins including lipase, haemolysin-coregulated protein and nucleoside diphosphate kinase. Expression of these secreted proteins was upregulated at the transcriptional level. Stearyl alcohol also induced the synthesis of polyhydroxyalkanoate. Secretory protein EliA was required for all these responses of NT80 cells to stearyl alcohol. Accordingly, the effects of stearyl alcohol were significantly reduced in the *eliA* deletion mutant cells of NT80 (Δ*eliA*). The remaining concentration of stearyl alcohol in the culture supernatant of the wild-type cells, but not that in the culture supernatant of the Δ*eliA* cells, clearly decreased during the course of growth. These observed phenotypes of the Δ*eliA* mutant were rescued by gene complementation. The results suggested that EliA is essential for these cells to respond to stearyl alcohol, and that it plays an important role in the recognition and assimilation of stearyl alcohol by NT80 cells.

INTRODUCTION

In many micro-organisms, the expression of lipase can be stimulated by oils and fatty acids (Sztajer et al., 1992; de Maria et al., 2005; Deive et al., 2009). Various types of detergents, such as Tween (polyoxyethylene sorbitan), are also known to induce the expression of lipase in species such as *Burkholderia glumae* (Boekema et al., 2007). In *Ralstonia* sp. NT80, a betaproteobacterium, a large amount of lipase is produced when stearyl alcohol is added to the culture medium (Ushio et al., 1996). For example, in *Ralstonia* sp. NT80, the lipase activity induced by stearyl alcohol was approximately sevenfold higher than that induced either by olive oil or by soybean oil (Ushio et al., 1996; Akanuma et al., 2013). This high induction of lipase expression by stearyl alcohol in *Ralstonia* sp. NT80 requires the secretory protein EliA, whose orthologues are widely distributed among the *Proteobacteria*, especially in the *Betaproteobacteria* and *Gammaproteobacteria* (Akanuma et al., 2013). Consistent with the above observation, the transcription level of the secretory lipase gene, lipA, in mutant NT80 cells lacking EliA (Δ*eliA*) was significantly reduced compared with that in the wild-type NT80 cells when these cells were induced with fatty alcohols, such as stearyl alcohol (Akanuma et al., 2013). However, why EliA is required for

**Abbreviations:** PHA, polyhydroxyalkanoate; PHB, poly(3-hydroxybutyrate); RT-PCR, reverse transcriptase PCR; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the draft genome sequence of *Ralstonia* sp. NT80 are BBQS01000001–BBQS01000176.

One supplementary figure and two supplementary tables are available with the online Supplementary Material.
the induction of lipA expression by fatty alcohols and whether EliA plays any role in cellular function(s) other than the induction of secretory lipase production remain unclear.

Secreted proteins play important roles in the adaptation of a bacterium to changing environmental conditions, including nutrient acquisition, stress protection and development of host–microbe associations via the formation of biofilms for cellular adhesion (Wandersman 1989; Tseng et al., 2009; Chater et al., 2010; Ciprandi et al., 2013). Proteobacteria have six types of protein secretion systems – types I–VI. Many secreted proteins are exported across the inner and outer membranes in a single step via the type I, type III, type IV or type VI secretion system. Other secreted proteins are first exported into the periplasmic space via the universal Sec or twin-arginine (Tat) pathway and are then translocated across the outer membrane via the type II or type V secretion system, or less commonly, via the type I or type IV secretion system (Tseng et al., 2009). Protein secretion is induced by various environmental changes such as quorum sensing, salt stress and addition of alcohol (Pumirat et al., 2009; Büttner & Bonas, 2010; Lu et al., 2012).

Polyhydroxyalkanoates (PHAs), such as poly(3-hydroxybutyrate) (PHB), are synthesized and accumulated as intracellular granules in several bacterial genera, including Ralstonia, Pseudomonas and Bacillus sp. (Tanadchangseng et al., 2009; Lopes et al., 2011; Raberg et al., 2011). This polymer is formed in the presence of excess supply of carbon and/or in the absence of one or more essential nutritional elements, and it can account for as much as 85 % of the cell dry weight under optimized growth conditions (Yamada et al., 2007; Jendrossek, 2009; Beeby et al., 2012). The synthesized PHA is reutilized when carbon and energy sources are depleted from the surrounding environment (Jendrossek & Handrick, 2002).

In a previous study, we showed that, in addition to lipase and EliA, many unidentified proteins were present in the culture supernatant of Ralstonia sp. NT80 cells grown in the presence of 1 % stearyl alcohol (Akanuma et al., 2013). This observation suggested that stearyl alcohol induces expression of many extracellular proteins and prompted us to investigate the multiple aspects of NT80 cells’ response to stearyl alcohol. Several previous studies reported the effects of methanol and ethanol on bacterial cells (Hendrickson et al., 2010; Lu et al., 2012) and several other studies examined the mechanism of assimilation of n-alkanes by bacteria (Bouchez Naïtal et al., 1999; Tani et al., 2001; Throne-Holst et al., 2006). The effects of fatty alcohols on bacterial cells, however, have hardly been explored. In the present study, to determine the effects of stearyl alcohol on NT80 cells, we compared secreted proteins from NT80 cells, grown with or without stearyl alcohol, by using 2D PAGE coupled with MALDI-TOF MS. The results of our comparative proteomic and transcriptional analyses showed that stearyl alcohol, besides inducing the expression of lipase, also induced expression of several other secreted proteins, which are regulated at the transcriptional level. Stearyl alcohol also induced the synthesis of PHA and affected the shapes of cells. In addition, we examined the responses of ΔeliA NT80 cells (lacking EliA) to stearyl alcohol and, based on the obtained results, discuss the possible functions of EliA.

METHODS

**Bacterial strains and growth conditions.** Ralstonia sp. NT80 (NT80) was isolated in our laboratory from soil as a producer of thermostable lipase (Ushio et al., 1996). The NT80 ΔeliA mutant (ΔeliA::gen and its complemented strain (ΔeliA::gen lipA::elA kan) were described previously (Akanuma et al., 2013). YP, a nutrient-rich medium used for culturing Ralstonia strains, was described previously (Akanuma et al., 2013). Strains were precultured at 30 °C for 16 h in 2 ml of YP medium in a test tube with reciprocal shaking (160 r.p.m.). Subsequently, 50 ml of YP medium was inoculated with 500 μl of this culture in a 500 ml baffled flask and the culture was incubated at 30 °C with reciprocal shaking (160 r.p.m.). Stearyl alcohol (Wako) was added to the growth medium to a final concentration of 1 % (w/v) as and when required.

**Preparation of extracellular proteins.** After the cells were removed by centrifugation at 8000 g for 10 min, 300 ml of the resulting culture supernatant was filtered. An equal volume of 20 % trichloroacetic acid was added to the supernatant, and the mixture was chilled at −20 °C for 20 min. Precipitated proteins were then harvested by centrifugation at 8000 g for 10 min at 4 °C. The protein pellet was washed with acetone and collected in a similar manner by centrifugation. The resulting pellet was air-dried briefly and suspended in 300 μl of denaturing buffer that contained 7 M urea, 2 M thiourea, 4 % CHAPS, 50 mM Tris (pH 8.8), 1 mM EDTA, 50 mM DTT and 2 mM PMSF. The amount of extracellular protein was quantified using a 2-D Quant kit (Amersham) with BSA as a standard. For 2D PAGE analysis, extracellular proteins were further processed using a 2D Clean-Up kit (GE Healthcare) following the manufacturer’s instructions. The resultant precipitate obtained from the 2D Clean-Up kit was resuspended in the urea buffer, which contained 8 M urea, 10 mM Tris (pH 8.0) and 1 mM DTT.

**Draft genome analysis.** The genomic DNA of Ralstonia sp. NT80 was extracted by the lysozyme–SDS–proteinase K method (Neumann et al., 1992), and the resultant DNA was treated with RNaseA and then extracted with phenol/chloroform to remove contaminating proteins. A DNA library with a median insert size of 500 bp was constructed for multiplexed paired-end read sequencing according to the protocol provided by Illumina. The final product was validated by using an Agilent Bioanalyser 2100 (Agilent). This library was sequenced on an Illumina Genome Analyser IIx following the manufacturer’s protocol, generating 100 bp paired-end reads and 6 bp index tags. The quality of the sequencing library was assessed by using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and the reads were trimmed to 80 bp. After trimming the reads, de novo sequence assembly was performed using Velvet assembler (Zerbino & Birney, 2008) and assembly parameters were optimized by using the VelvetOptimizer (http://www.bioinformatics.net.au/software.velvetoptimiser.shtml). The assembled contigs were annotated by the Microbial Genome Annotation Pipeline server (MiGAP; http://www.migap.org/). The encoded amino acid sequences of the predicted genes exhibiting high alignment length (over 60 % of own length) and match identity (over 60 %) were chosen, and the best hit showing highest alignment length percentage and match identity was assigned as the annotation of the predicted gene. In silico Molecular
Cloning Genomics Edition (IMCGE) (In silico Biology) was used to analyse annotated contigs. The draft genome sequence of *Ralstonia* sp. NT80 has been deposited in the DDBJ/EMBL/GenBank databases under accession numbers BBQ501000001–BBQ501000176.

**2D gel electrophoresis.** Isoelectric focusing was performed using the Immobiline DryStrip (pH 4–7; GE Healthcare). Extracellular fractions containing 300 mg of proteins were loaded into the dry strip by rehydration, which was carried out for 18 h in a solution that contained 8 M urea, 4 % (w/v) CHAPS, 65 mM DTT, a trace of bromophenol blue, 0.5 % (v/v) IPG buffer (pH 4–7; GE Healthcare) and 1.2 μL of DeStreak Rehydration solution (GE Healthcare). Isoelectric focusing was carried out using the NA-1410R electrophoresis device for dry strips (Nihon Eido) and employing the following voltage profile: 500 V for 4 h, 700 V for 1 h, 1000 V for 1 h, 2000 V for 1 h and 3000 V for 16 h. After isoelectric focusing, the DryStrip gel was soaked in the equilibration buffer, which contained 0.5 M Tris (pH 6.8), 6 M urea, 10 % (v/v) glycerol, 1 % SDS, 15 mM DTT and a trace of bromophenol blue, for 30 min; the equilibrated gel was then carefully placed onto a 15 % SDS-polyacrylamide gel for the separation of proteins in the second dimension, and gel electrophoresis was carried out using Laemmli’s Tris-glycine buffer (Laemmli, 1970). Following electrophoresis, the gel was stained with Coomasie brilliant blue R-250.

**Protein identification by MS.** Protein spots were identified as described previously (Akanuma *et al.*, 2009). Briefly, protein spots, excised from gels, were reduced, alkylated and then hydrolysed with modified trypsin (Promega) at 37 °C for 18 h. Peptides were extracted from the gel pieces, desalted using ZipTipC18 pipette tips (Millipore), and then eluted with 50 % acetonitrile and 0.1 % trifluoroacetic acid. Samples for MALDI-TOF analysis were prepared by mixing the eluate with an equal amount of 2-cyano-4-hydroxycinnamic acid. MALDI-TOF MS spectra were recorded using a Shimadzu AXIMA-CN mass spectrometer (Shimadzu). MALDI-TOF peptide mass fingerprint data were compared with the *Ralstonia* sp. NT80 sequence database using the MASCOT search engine (Matrix Science), and proteins were identified using the probability-based MOWSE score algorithm. Search parameters used for this analysis were as follows: mass accuracy, 300 p.p.m.; missed cleavage, none; fixed modification, carboxymethylation of Cys residues; variable modification, oxidation of Met residues. All proteins identified in this study had MOWSE scores > 50 (a MOWSE score > 50 guarantees identification of the protein with > 95 % probability).

**RNA extraction and quantitative RT-PCR.** Total RNA extraction from NT80 cells and quantitative reverse transcriptase PCR (RT-PCR) were performed as described previously (Akanuma *et al.*, 2013). The cDNA was synthesized from 1 μg of DNase-treated RNA using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) in accordance with the manufacturer’s instructions. All reactions were carried out using a KAPA SYBR FAST Master Mix (KAPA Biosystems) reaction mixture on an MyiQ2 Real-Time PCR system (Bio-Rad) and using the following reaction conditions: 10 s at 95 °C, followed by 40 cycles of 5 s at 95 °C for denaturation and 30 s at 62 °C for annealing and extension. To amplify the internal regions of the target genes, we used appropriate combinations of PCR primers (listed in Table S1, available in the online Supplementary Material). All reactions were performed in triplicate, and the resulting data were normalized using the amount of 16S rRNA as the internal standard.

**Biofilm formation and crystal violet staining.** For biofilm formation, cells were precultured at 30 °C for 16 h in 2 ml of YP in the absence of stearyl alcohol with reciprocal shaking. Subsequently, 20 μl of the culture was used to inoculate 2 ml of YP medium (in a new test tube) without or with 1 % stearyl alcohol. The culture was incubated at 30 °C without shaking for 24 h. After removing the culture, the test tube was rinsed with distilled water and the biofilm was stained with 2 ml of 1 % crystal violet for 1 h as described previously (O’Toole & Kolter 1998). The stained biofilm was rinsed twice with 2 ml of distilled water to remove the unbound crystal violet. Finally, the crystal violet that remained bound to the biofilm was dissolved in 1 ml of 95 % ethanol and its amount was quantified by measuring the absorbance at 595 nm.

**Transmission electron microscopy (TEM).** After growing cells for 72 h, the culture was centrifuged at 8000 g for 10 min, and the collected cells were sandwiched immediately between copper discs and quickly frozen in liquid propane at −175 °C. Frozen samples were freeze-substituted with 2 % osmium tetroxide in acetone and 2 % distilled water at −80 °C for 48 h, and then kept at −20 °C for 2 h followed by warming to 4 °C for 1 h. Samples were first dehydrated by incubating them in anhydrous acetone (three times, 30 min each). Dehydrated samples were mixed with a 70 : 30 mixture of anhydrous acetone and Quetol-651 resin (Nissin EM) for 1 h, following which the acetone was volatilized by keeping the cap of the tube open overnight. Samples were transferred to a fresh 100 % resin, and polymerized at 60 °C for 48 h. The resulting blocks were cut into ultrathin sections (70 nm) with a diamond knife using an ultramicrotome (Ultrcut, UCT; Leica). Cut sections were placed on copper grids, stained with 2 % uranyl acetate at room temperature for 15 min, rinsed with distilled water and then secondarily stained with lead stain solution (Sigma Aldrich) at room temperature for 3 min. The stained sections were observed under a transmission electron microscope (JEM-1200EX; JEOL) at an acceleration voltage of 80 kV. Digital images (2048 × 2048 pixels) were captured with a CCD camera (Velveta; Olympus Soft Imaging Solutions).

**Quantification of PHA.** The amount of PHA produced by NT80 cells was quantified as described previously (Shakeri *et al.*, 2012). In brief, cells were harvested from 1 ml of culture and washed with 1 ml of PBS buffer (pH 7.0). The cell pellet was incubated with 1 % (v/v) Triton X-100 solution for 30 min at room temperature. After centrifugation, the pellet was first washed with PBS buffer, and then incubated with 1 ml of alkaline sodium hypochlorite solution for 1 h at 30 °C. The mixture was centrifuged and the pellet was sequentially washed with water, alcohol and acetone. The pellet was incubated with 1 ml of concentrated sulfuric acid at 100 °C for 10 min. After cooling, the amount of crotonic acid formed was determined by measuring the absorbance of the resulting solution at 235 nm using sulfuric acid as a blank. The calibration curve was determined by using pure PHB.

**Quantification of stearyl alcohol in the culture supernatant.** Stearyl alcohol remaining in the culture supernatant of cells grown in the medium containing 1 % stearyl alcohol was isolated by first extracting 1 ml of the supernatant with 500 μl diethyl ether (three times) and then exsiccating the resulting liquid using a rotary evaporator. The pellet containing stearyl alcohol was resuspended in an appropriate volume of diethyl ether and analysed by GC-MS using a Shimadzu GC-2014 instrument equipped with a flame ionization detector and a capillary column (DB-5, 30 m × 0.25 mm; Agilent Technologies). Cetyl alcohol was used as an internal standard to estimate the yield of stearyl alcohol. A calibration curve was established by using pure stearyl alcohol.

**RESULTS**

**Effects of stearyl alcohol on cell viability and extracellular protein expression.** We first examined the effects of adding stearyl alcohol to the medium on cell growth and cell viability. When NT80 cells were grown in PY medium at 30 °C, cells entered stationary...
were measured at the times indicated by plating diluted cultures on PY agar. (b) Quantification of stearyl alcohol remaining in the culture supernatants of wild-type, ΔeliA and ΔeliA complemented cells. Stearyl alcohol remaining in the culture supernatants was extracted at the times indicated and its amount was quantified by GC as described in Methods. (c) Stearyl alcohol affects the amount of extracellular protein. Extracellular protein concentration was measured as described in Methods. Means of six (a) or three (b and c) independent experiments are shown. Error bars indicate standard deviations. Strain ΔeliA : : gen tlpA : : eliA kan is described as ΔeliA complemented cells.

An overview of extracellular proteins detected in the culture supernatant of NT80 cells

To detect as many stearyl-alcohol-induced secreted proteins as possible, protein samples prepared from the culture supernatant of NT80 cells grown in the absence and presence of stearyl alcohol for 72 h were analysed by 2D PAGE (Figs 2 and S1). We chose to use the 72 h growth phase at about 12 h after inoculation. Addition of stearyl alcohol to the medium did not affect cell growth in the exponential phase, but it moderately improved the viability of cells in the late stationary phase, although relatively large standard deviations were seen (Fig. 1a). Although the number of viable cells at 24 h after inoculation was not significantly different in cultures grown in the presence or absence of stearyl alcohol at the late stationary phase the viable cell count in culture grown in the absence of stearyl alcohol was lower than that in culture grown in the presence of 1% stearyl alcohol (Fig. 1a). More importantly, the number of viable cells barely decreased in culture grown in the presence of stearyl alcohol even at 72 h after inoculation. It is possible that NT80 cells might utilize stearyl alcohol as a carbon source. Therefore, we next used GC to determine the amount of stearyl alcohol remaining in the culture supernatant of NT80 cells and found that the amount of stearyl alcohol in the culture supernatants of wild-type and ΔeliA complemented NT80 cells, but not in the culture supernatant of ΔeliA NT80 cells, decreased with growth time (Fig. 1b). These results suggested that the NT80 cells might have maintained their viability by utilizing stearyl alcohol, possibly as a carbon source. Stearyl alcohol also induced accumulation of proteins in the culture supernatant. As shown in Fig. 1(c), the amount of protein found in the culture supernatants of wild-type and ΔeliA complemented cells, but not in the culture supernatant of ΔeliA cells, all of which were grown in the presence of stearyl alcohol, increased significantly during the late stationary phase, whereas such an increase in the protein amount was not observed in the culture supernatants of both wild-type and ΔeliA cells grown in the absence of stearyl alcohol. These results suggest that the addition of stearyl alcohol to the growth medium of NT80 cells partially maintained their viability and induced expression of extracellular proteins as well.
condition for this experiment because, as shown in Fig. 1(c),
the amount of extracellular proteins found in the culture
supernatant of NT80 cells increased at least up to 72 h
after inoculation. To identify the protein spots detected
on the 2D gels, we next performed peptide mass fingerprinting analysis. For this, we first generated a draft
genome sequence of the *Ralstonia* sp. NT80, as its
genome sequence was not known. Sequence analysis of
the genomic DNA of *Ralstonia* sp. NT80 provided a draft
genome of 5.8 Mb in 176 contigs (with the largest 29 com-
prising >99% of the total), and the genome was found to
contain 5301 ORFs. When extracellular proteins prepared
from the culture supernatant of wild-type cells grown in
the presence of stearyl alcohol were separated by 2D PAGE,
more than 60 protein spots were visualized on the gel,
out of which 30 proteins were identified (Figs 2 and S1).
In contrast, approximately only 20 protein spots were
visualized on the 2D gels of extracellular proteins pre-
pared from the culture supernatants of cells grown in the
abscense of stearyl alcohol, out of which 16 proteins were
identified (Fig. S1, Table S2). Ten out of 30 proteins, identi-
fied from the stearyl-alcohol-induced cells, were predicted
to be extracellular (secreted) proteins (Table S2)
by the PSORTb program (http://www.psort.org/psortb/).
Thus, out of 66 predicted extracellular proteins encoded
by the NT80 genome, in this study we identified 10 pro-
teins (about 15%) that were secreted from these cells
when induced with stearyl alcohol. Out of the 10 identified
secreted proteins (listed in Table S2), only four proteins
(EliA, LipA, peptidase S8/S53 subtilisin kexin sedolisin
and tip pilus assembly protein tip-associated adhesin
PilY1-like protein) contained a putative N-terminal signal
peptide sequence, suggesting that they could be secreted
by the type II secretion system (T2SS); the following six
proteins, although predicted to be extracellular, did not
have any known N-terminal signal peptide sequences
for secretion (Table S2): Hcp (haemolysin-coregulated
protein), Ndk (nucleotide diphosphate kinase), lipopro-
tein, flagellin domain protein, FlgK and FlgE. Among
these six proteins, Hcp forms the extracellular part of
the type VI secretion machinery and is released into the culture
medium by T6SS (Mougous et al., 2006; Records, 2011;
Decoin et al., 2014). Ndk (nucleoside diphosphate kinase)
and the flagellum proteins FlgK and FlgE, by contrast, are
secreted by the T3SS (Macnab, 2004; Neeld et al., 2014).
However, we could not predict the pathway by which the
lipoprotein (ORF No. 5154) is secreted. Among the 30
identified proteins, 13 were predicted to be cytoplasmic
(Table S2). These putative cytoplasmic proteins were prob-
ably released into the culture supernatant due to cell lysis.
A number of previous studies have also identified cyto-
plasmic proteins in the culture supernatant of proteobacte-
rial cells (Wehmhöner et al., 2003; Goo et al., 2010;
Mariappan et al., 2011).

### Stearyl alcohol induces expression of several secretory proteins

To verify the effect of stearyl alcohol on the expression of
secretory proteins, we compared proteins found in the cul-
ture supernatant of NT80 cells grown without or with stearyl
alcohol by 2D gel electrophoresis (Fig. 2). Five of the iden-
tified proteins, as listed in Table 1, were predicted to be extracellu-
lar. Out of these five proteins, expression of Ndk and
lipoprotein was detected only in the culture supernatant of
stearyl-alcohol-induced cells and expression of EliA, LipA
and Hcp was detected in the culture supernatant of both
uninduced and stearyl-alcohol-induced cells, but expression
levels of the latter proteins clearly increased in the culture
supernatant of induced cells. We have previously reported

![Fig. 2. 2D gel separation of proteins obtained from the culture supernatant of the wild-type NT80 cells grown in the absence (left panel) or presence (middle panel) of stearyl alcohol and NT80 ΔeliA cells grown in the presence of stearyl alcohol (right panel). In the first dimension, proteins were separated using a pH gradient of 4–7. Numbers correspond to the protein spots, which were identified by MALDI-TOF MS (see Tables 1 and S2). Dashed circles without any number indicate the positions of protein spots that either were not identified or whose abundances were clearly lower than those in the culture supernatants of wild-type cells grown in the presence of stearyl alcohol, wild-type cells grown in the absence of stearyl alcohol or ΔeliA cells grown in the presence of stearyl alcohol. Protein spot indicated with ‘P’ in the right panel corresponds to porin (ORF no. 0502).](image-url)
that the expression of two of these secretory proteins, lipase and EliA (a protein that promotes the stearyl-alcohol-induced expression of lipase), was induced by stearyl alcohol (Akanuma et al., 2013). Two other secretory proteins in this list were Hcp and Ndk. The Hcp identified in this study shared 95 and 71 % identity with the haemolysin-coregulated protein of Ralstonia solanacearum PSI07 and Burkholderia pseudomallei 1026b, respectively (Remenant et al., 2010; Hayden et al., 2012), whereas the Ndk identified in this study shared 97 and 86 % identity with the nucleoside diphosphate kinase of R. solanacearum PSI07 and B. pseudomallei 1026b, respectively (Remenant et al., 2010; Hayden et al., 2012). Of these two proteins, Hcp has been shown to be an extracellular part of the T6SS (Mougous et al., 2006; Records, 2011; Decoin et al., 2014), which has been reported to be involved in secreting virulence-related proteins either out of or directly into the cells of a eukaryotic host (Schell et al., 2007; Burtnick et al., 2011). Secretory Ndk, by contrast, has been shown to be exported via the T3SS and is known to be involved in the virulence of pathogenic bacteria such as Pseudomonas aeruginosa and Mycobacterium tuberculosis (Chakrabarty, 1998; Neeld et al., 2014).

Table 1. Stearyl-alcohol-induced expression of major extracellular proteins

<table>
<thead>
<tr>
<th>Spot no.*</th>
<th>ORF no.</th>
<th>Gene product</th>
<th>Cleavage site†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5152</td>
<td>EliA</td>
<td>25/26</td>
</tr>
<tr>
<td>2</td>
<td>5214</td>
<td>LipA (triaclylglycerol lipase)</td>
<td>46/47</td>
</tr>
<tr>
<td>3</td>
<td>0052</td>
<td>Hcp1 (T6SS effector)</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>4226</td>
<td>Ndk (nucleoside diphosphate kinase)</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>5154</td>
<td>Lipoprotein</td>
<td>None</td>
</tr>
</tbody>
</table>

*Spot numbers refer to the respective protein spot numbers on 2D gels shown in Figs 2 and S1.
†Cleavage site for the N-terminal signal peptide was predicted using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/).

To determine whether expression of these secreted proteins was induced at the transcriptional level by stearyl alcohol, mRNA levels of these proteins were analysed by quantitative RT-PCR. Comparison of mRNA levels of ndk, hcp and lipA in cells grown in the absence and presence of stearyl alcohol revealed that their mRNA levels increased significantly during growth after stearyl alcohol was added to the medium (Fig. 3a). In contrast, mRNA of the gene encoding peptidase S8/SS3 subtilisin kexin sedolisin, which is a secreted protein, was not induced by stearyl alcohol (Fig. 3a). These results demonstrated that stearyl alcohol induced expression of several secretory proteins at the transcriptional level.

Expression of several proteins also decreased when stearyl alcohol was added to the growth medium. One of the protein spots on the 2D gel (spot no. 8, Fig. 2), expression of which significantly decreased upon the addition of stearyl alcohol, was identified as the flagellin domain protein. This observed decrease in the expression of flagellin domain protein might be due to the induced expression of a protease that specifically degraded the flagellin domain protein. Although a candidate protease remains yet to be identified, it is known that lack of Lon protease increases the stability of flagellin in the proteobacterium Proteus mirabilis (Clemmer & Rather, 2008). An alternative explanation would be that addition of stearyl alcohol to the growth medium inhibited the expression of flagellin domain protein. At present, however, we do not have any experimental data that would support one of these two alternative possibilities.

**Fig. 3.** Expression ratios of mRNAs of secreted proteins were determined by using quantitative RT-PCR at the indicated times and expressed as a fold change: (a) wild-type cells induced with stearyl alcohol/uninduced wild-type cells; (b) ΔeliA cells induced with stearyl alcohol/uninduced ΔeliA cells (12–36 h); and ΔeliA complemented cells induced with stearyl alcohol/uninduced ΔeliA complemented cells (36 h). Means from three experiments are shown (error bars, SD).
Stearyl alcohol induces PHA production

To determine whether stearyl alcohol has any effect on the morphology of NT80 cells, we examined NT80 cells grown in the presence or absence of stearyl alcohol using TEM. Interestingly, addition of stearyl alcohol to the medium affected the organization of the cell membrane, as we observed granules with diameters of 100–200 nm only in wild-type cells, but not in ΔeliA cells, that were grown in the presence of stearyl alcohol (Fig. 4a). Note that samples for TEM were prepared by rapid freezing followed by freeze-substitution fixation to eliminate artefacts.

Ralstonia eutropha, which is closely related to NT80, is known to accumulate PHB granules in cells grown in the presence of excess carbon supply and/or in the absence of one or more essential nutritional elements (Jendrossek, 2009). Therefore, we presumed that the observed granules in NT80 cells might be PHAs because of their shapes and sizes. Consistent with this idea, our analysis showed that PHA was accumulated only in NT80 cells grown in the presence of stearyl alcohol (Fig. 4b). Thus, addition of stearyl alcohol induced PHA production and affected the organization of the cell membrane (Fig. 4). Although the composition of the PHA granule has been reported to be altered depending on the carbon source (Tsuge, 2002), in this study we assumed that the observed granules in the NT80 cells were made of PHAs, and therefore we used PHB to establish the calibration curve. It has been reported that PHA influences biofilm formation (Tribelli & López, 2011). Although PHA is known to play an important role in cell survival, biofilm formation is also valuable for maintaining the viability of bacterial cells in a stressful environment (Ruiz et al., 2001; Webb et al., 2003; Ratcliff et al., 2008). Thus, we next examined the effects of stearyl alcohol on biofilm formation. When stearyl alcohol was added to the growth medium, the amount of biofilm produced by cells 24 h after inoculation was significantly increased in wild-type and ΔeliA complemented cells, but not in ΔeliA cells (Fig. 5). Therefore, stearyl alcohol not only affected...
cell shape, but also induced PHA production and biofilm formation.

**EliA is required for the responses of NT80 cells to stearyl alcohol**

As described above, addition of stearyl alcohol to the growth medium affected NT80 cells in various ways. We have previously found that lipase expression was greatly reduced in cells lacking EliA (Akanuma et al., 2013). Thus, to investigate whether EliA is required for the responses observed in NT80 cells grown in the presence of stearyl alcohol, we repeated these studies using a ΔeliA NT80 strain. Interestingly, the stearyl-alcohol-induced expression of secreted proteins was greatly reduced in these EliA-lacking cells (Figs 1c and 2). In particular, transcription of ndk, hcp and lipA, induced by stearyl alcohol, were significantly reduced in the ΔeliA mutant (Fig. 3b). The amount of putative flagellin protein (spot no. 8 in the 2D gel) was also decreased in the ΔeliA cells (Fig. 2), although at present we do not have any logical explanation for this observation. Furthermore, addition of stearyl alcohol did not induce PHA production and biofilm formation in these cells (Figs 4 and 5). In the absence of any added stearyl alcohol, the viability of ΔeliA cells during the stationary phase also decreased, as was observed for the wild-type NT80 cells; however, when the culture medium contained stearyl alcohol, this decrease in viability, which was virtually suppressed in the wild-type NT80 cells, was slightly attenuated in ΔeliA NT80 cells (Fig. 1a). To verify that these phenotypes were due solely to the lack of EliA, genetic complementation tests were performed. As expected, expression of EliA in the ΔeliA cells by genetic complementation caused these cells to respond to stearyl alcohol in a manner similar to that of the wild-type NT80 cells (compare the results shown in Figs 1, 3b, 4b and 5). Examination of ΔeliA cells by TEM revealed that the addition of stearyl alcohol to the medium did not have any effect on the cell membrane of ΔeliA cells (Fig. 4a, right panel), which is in contrast to what was observed for the wild-type NT80 cells, where we observed granule formation when stearyl alcohol was added to the medium (Fig. 4a, middle panel). We previously predicted that EliA is involved in recognition and/or incorporation of fatty alcohols, such as stearyl alcohol (Akanuma et al., 2013). In the present study, we determined the amount of stearyl alcohol remaining in the culture supernatants of wild-type and ΔeliA NT80 cells by GC. We found that the amount of stearyl alcohol in the culture supernatant of wild-type NT80 cells decreased with cultivation time, whereas the amount of stearyl alcohol in the culture supernatant of ΔeliA cells remained the same (Fig. 1b). It is noteworthy that the rate of decrease in stearyl alcohol concentration in the culture supernatant of ΔeliA complemented NT80 cells was almost same as that of the wild-type NT80 cells (Fig. 1b). Thus, these results clearly demonstrated that EliA plays a vital role in the recognition and assimilation of stearyl alcohol. Taken together, our results suggest that EliA is required for the responses of NT80 cells to stearyl alcohol.

**DISCUSSION**

In the present study, we have characterized the responses of *Ralstonia* sp. NT80, a betaproteobacterium, to stearyl alcohol. Comparative proteomic and transcriptional analysis revealed that stearyl alcohol induced expression of several secreted proteins, and that this induction occurred at the transcriptional level. In addition, we found that stearyl alcohol promoted the synthesis of PHA and altered the organization of the cell membrane. Our results also suggest that NT80 cells required the secretory protein EliA to exhibit various responses to stearyl alcohol.

Among the proteins successfully identified from the 2D gel, abundances of five proteins, all of which were predicted to be extracellular, clearly increased when the wild-type cells were grown in the presence of stearyl alcohol (Fig. 2, Table 1). Expression levels of transcripts of *ndk*, *hcp* and *lipA* were also upregulated by stearyl alcohol (Fig. 3a). In the natural environment, land plants use fatty alcohols, such as palmityl and stearyl alcohols, to form waxes, which provide protection against desiccation, bacterial attacks and UV radiation (Buschhaus & Jetter, 2011; Isaacson et al., 2009). Lipases (EC 3.1.1.3) catalyse hydrolysis not only of glycerides formed from glycerol and long-chain fatty acids but also of various esters including polyesters. In particular, a lipase purified from the extracellular fraction of NT80 cells hydrolysed esters of long-chain fatty acids (our unpublished data). Given that *Ralstonia* sp. NT80 was isolated from soils that contained fallen plant leaves, it is therefore possible that soil bacteria, such as NT80, may recognize fatty alcohols present in the dead leaves and other dead tissues of plants and consequently may secrete large amounts of lipase to hydrolyze the waxy plant polymer cutin, the most abundant structural component of the plant cuticle, which is composed of covalently cross-linked C16 or C18 oxygenated fatty acids and glycerol (Pollard et al., 2008; Beisson et al., 2012), and obtain a source of carbon. Hcp, another secretory protein identified in this study, is known to form a hexameric ring and is postulated to be an extracellular translocon of the T6SS (Mougous et al., 2006; Records, 2011; Decoin et al., 2014). It was reported earlier that a T6SS-defective mutant of *R. solanacearum* showed significantly attenuated virulence in tomato (Zhang et al., 2014). In addition, deletion of *hcp* has been shown to reduce the tumorigenic efficiency of *Agrobacterium tumefaciens* on potato tuber discs (Wu et al., 2008). The secretory lipase has also been shown to be a virulence factor of *Xanthomonas oryzae pv. oryzae* and *B. glumae*, which are pathogenic to rice plants (Aparna et al., 2009; Ham et al., 2011). While the function of Ndk in the virulence of phytopathogenic bacteria remains unclear, it is known that secretory Ndk is involved in the virulence of *Pseudomonas aeruginosa* and *M. tuberculosis*, which are pathogenic to humans (Chakrabarty, 1998;
Kim et al., 2014). Although a eukaryotic host cell for Ralstonia sp. NT80 has yet to be identified, several species belonging to the genera Ralstonia and Burkholderia are known to infect plant cells (Cottyn et al., 1996; Jeong et al., 2003; Coenye et al., 2003). As described above, land plants use fatty alcohols, including stearyl alcohol, in the form of waxes (Buschhaus & Jetter, 2011). These waxes are not only present in the plant leaf but also present in the plant root (Li et al., 2007). Phytopathogenic bacteria, such as R. solanacearum, interact with and invade host plants from roots (Peeters et al., 2013). Assuming that Ralstonia sp. NT80 could potentially cause disease in plants, its putative virulence proteins are probably induced by the fatty alcohols naturally found in the plant waxes. In this regard, it is noteworthy that the expression of the orthologues of ndk, hcp and lipA is induced by quorum sensing (Goo et al., 2010; Records, 2011). Therefore, it is possible that these genes, in addition to being induced by stearyl alcohol, were also induced by quorum sensing, as addition of stearyl alcohol to the growth medium partially maintained cell viability. However, expression of these genes was already induced at the transcriptional level even at 24 h post-inoculation when stearyl alcohol did not affect cell viability (i.e., cell density) (Figs 1a and 3a). Therefore, it is most likely that expression of ndk, hcp and lipA was induced by quorum sensing as well as by a signal that can be attributed to stearyl alcohol. We have also identified several cytoplasmic proteins, such as S-adenosyl-l-homocysteine hydrolase (spot no. 21, Fig. S1) and elongation factor Tu (spot no. 23, Fig. S1), in the culture supernatant of NT80 cells grown in the presence of stearyl alcohol (Table S2). These results suggested that expression of these cytoplasmic proteins is also induced by stearyl alcohol, as they were found in the culture supernatant of cells grown in the presence of stearyl alcohol (Fig. S1, bottom panel) but not in the culture supernatant of cells grown in the absence of stearyl alcohol (Fig. S1, top panel); they were found among the extracellular proteins probably because of cell lysis. However, we have not performed any experiment to confirm that expression of these cytoplasmic proteins was indeed induced by stearyl alcohol. At present, we also do not know whether stearyl alcohol actually induced cell lysis, but our results clearly suggested that stearyl alcohol helped in maintaining cell viability. Nonetheless, at least five proteins, listed in Table 1, were secreted by NT80 cells after their expression was upregulated by stearyl alcohol. Consistent with this observation, the signal peptides of LipA and EliA, both of which were detected in the culture supernatant of cells grown in the presence of stearyl alcohol, were found to be cleaved (Akanuma et al., 2013, and our unpublished data).

Addition of stearyl alcohol to the medium also induced PHA production (Fig. 4). Genes responsible for PHA biosynthesis in R. eutropha have been cloned and characterized; they comprised a β-ketothiolase (phaA), an acetoacetyl-CoA reductase (phaB) and a PHA synthase (phaC) (Peoples & Sinskey, 1989a, b). The NT80 genome also has an operon encoding homologues of PhaC, PhaA and PhaB. Transcription of the operon responsible for the PHA biosynthesis in NT80 cells was, however, not significantly upregulated by stearyl alcohol (results not shown). In this regard, it is noteworthy that the transcription level of the phaCAB gene cluster of R. eutropha did not change significantly under the conditions where PHA is produced or utilized (Brigham et al., 2012). Under nitrogen-deficient conditions, the biosynthesis of PHA in Paracoccus denitrificans is also not regulated at the mRNA or protein level, but depends on the amount of acetyl-CoA (Kojima et al., 2004). In Gram-negative bacteria, fatty alcohols are converted to fatty acids via a pathway that involves an alcohol dehydrogenase (AlkJ) and an aldehyde dehydrogenase (AlkH). The produced fatty acid then serves as a substrate for acyl-CoA synthetase (AlkK) and the resulting acyl-CoA enters the β-oxidation pathway to be converted to acetyl-CoA (Van Hamme et al., 2003; Wentzel et al., 2007). As fatty alcohols are terminally converted to acetyl-CoA, as described above, the amount of acetyl-CoA accumulated in the NT80 cells following the assimilation of stearyl alcohol probably exceeded its metabolic degradation via the TCA cycle at the stationary phase, and thereby promoted PHA biosynthesis. Other than its role as an energy reserve, PHA also plays important roles in stress resistance and survival of bacterial cells living in low nutrient environments (Ruiz et al., 2001; Ratcliff et al., 2008). Therefore, the production of PHA in the NT80 cells may help in maintaining cell viability during the stationary phase, at least in part, when cells were grown in the presence of stearyl alcohol (Figs 1a and 4). Recently, we performed proteomic analysis of the NT80 membrane fraction and found that stearyl alcohol induced expression of OmpW family proteins (unpublished data). OmpW is involved in protecting bacteria from various environmental stresses (Nandi et al., 2005; Gil et al., 2007). Thus, it is possible that stearyl-alcohol-induced OmpW expression may also help in maintaining cell viability. However, the viability of the ΔelA mutant cells, in which stearyl alcohol did not induce PHA accumulation (this study) and expression of OmpW (unpublished data), marginally improved when stearyl alcohol was added to the medium (Fig. 1a). Therefore, in addition to inducing PHA production and OmpW expression, stearyl alcohol maintains cell viability probably by some other unknown mechanism(s).

Examination of NT80 cells by TEM revealed that stearyl alcohol affected organization of the cell membrane (Fig. 4a). The fluidity of the bacterial membrane is known to be altered by medium composition, quorum sensing and stress responses (Bayse et al., 2005). The effect of fatty alcohols on membrane fluidity has been studied in the gammaproteobacterium Acinetobacter calcoaceticus, in which cells grown in the presence of fatty alcohol had increased amounts of unsaturated fatty acids, containing bent acyl chains, incorporated into their cell membranes, causing the membranes to expand (Kabelitz et al., 2003). This might be one of the reasons why stearyl alcohol affected the organization of the cell membrane of
We have previously reported that EliA facilitates induction of lipase expression by stearyl alcohol (Akanuma et al., 2013). In the present study, we have found that EliA was required not only for inducing the expression of lipase but also for the multiple responses of NT80 cells to stearyl alcohol, such as expression of genes of secreted proteins Hcp and Ndk, and production of PHA. In agreement with our suggestion that the induced synthesis of PHA in the wild-type NT80 cells was caused by the assimilation of stearyl alcohol followed by accumulation of acetyl-CoA, we found that the amount of stearyl alcohol in the culture supernatant of the wild-type cells decreased during growth as PHA was accumulated, whereas the amount of stearyl alcohol in the culture medium of ΔeliA cells remained the same even in the late stationary phase, and granule formation was not observed in these cells (Figs 1b and 4). These results were consistent with the notion that EliA is required for PHA synthesis. It has also been reported that the protein-like activator protein of Pseudomonas aeruginosa PG201, a protein homologous to EliA, facilitates oxidation and is involved in the assimilation of n-hexadecane, which is similar to the long hydrocarbon-like chain of stearyl alcohol (Hisatsuka et al., 1972, 1977; Hardegger et al., 1994). Thus, EliA appears to be essential, at least, for the assimilation of stearyl alcohol by NT80 cells, and it may also play an important role in the survival of these cells during the stationary phase. Alternatively, the function of EliA in NT80 cells may be to recognize stearyl alcohol, because EliA was required for the stearyl-alcohol-induced expression of secreted proteins. Given that the transcription of eliA was upregulated earlier than the transcriptions of genes of other secreted proteins tested here (Fig. 3a), it may be possible that NT80 cells utilized the secreted EliA to recognize stearyl alcohol in the culture medium. If NT80 cells could indeed degrade plant cutin using the secreted lipase, expression of which is induced by fatty alcohols present in the plant waxes, then EliA may also have an important role in obtaining carbon sources from the surrounding environment. Thus, further studies would be necessary to unravel the pathway by which stearyl alcohol induces various cellular events and to understand the detailed function of EliA.

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REFERENCES


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