A Genome-wide Approach to Identify the Genes Involved in Biofilm Formation in *E. coli*

Emma Tabe Eko Niba1, Yoshiaki Nakamura1, Megumi Nagase2, Hirotada Mori3 and Madoka Kitakawa2,*

Graduate School of Science and Technology, Kobe University, Kobe, Hyogo 657-8501, Japan1, Faculty of Science, Department of Biology, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe, Hyogo 657-8501, Japan2 and Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan3

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Abstract

Biofilm forming cells are distinctive from the well-investigated planktonic cells and exhibit a different type of gene expression. Several new *Escherichia coli* genes related to biofilm formation have recently been identified through genomic approaches such as DNA microarray analysis. However, many others involved in this process might have escaped detection due to poor expression, regulatory mechanism, or genetic backgrounds. Here, we screened a collection of single-gene deletion mutants of *E. coli* named ‘Keio collection’ to identify genes required for biofilm formation. Of the 3985 mutants of non-essential genes in the collection thus examined, 110 showed a reduction in biofilm formation nine of which have not been well characterized yet. Systematic and quantitative analysis revealed the involvement of genes of various functions and reinforced the importance in biofilm formation of the genes for cell surface structures and cell membrane. Characterization of the nine mutants of function-unknown genes indicated that some of them, such as *yfgA* that genetically interacts with a periplasmic chaperone gene *surA* together with *yciB* and *yciM*, might be required for the integrity of outer membrane.

Keywords: biofilm; deletion mutant; *E. coli*

1. Introduction

Bacteria have evolved elaborate mechanisms for adhering to and colonizing solid surfaces, thereby establishing microbial communities known as biofilms.1 These represent a distinct lifestyle for bacteria that provides protection from deleterious conditions, thereby raising various problems to our life such as causing persistent and chronic human infections2 or contamination of food products.

The transition from a planktonic to a sedentary biofilm mode of life requires the coordinated regulation of genes involved in the development of biofilms, which is an interesting theme to investigate the intricate network of signal transduction for gene expression in bacterial cells.3 The latter lifestyle would require the expression of genes that have not been investigated in studies with planktonic cells. Recent analyses of biofilms using DNA microarray4–6 revealed that hundreds of genes including many of uncharacterized are differentially expressed in biofilms, which would provide insights into the genetic basis for biofilm formation. However, the agreement for differential gene expression is limited among these studies, probably reflecting differences in experimental conditions as well as the nature of biofilm itself.7 The environment within the biofilm is heterogeneous and biofilm formation is a dynamic process.8 Furthermore, the different expression of some genes may be due to differences in growth of planktonic cells used as control.9 Genetic analysis revealed that surface structures such as flagella and specific outer-membrane adhesins, Type 1, and curli fimbriae of *Escherichia coli* are important for biofilm formation, though they are not indispensable.10,11 The extent of
biofilm formation and the effect of mutation seem to be variable depending on the strains used.12–14

Keeping the above problems in mind, we started identifying genes that cause upon disruption a reduced biofilm-formation in the same genetic background. In this study, we used a collection of single-gene deletion mutants of all non-essential genes of E. coli called ‘Keio collection’15 and performed a quantitative analysis of their biofilm formation.

2. Materials and methods

2.1. Bacterial strains and plasmid

Escherichia coli K-12 strain BW25113 and isogenic deletion mutants of the Keio collection15 were used. KR0401 is a derivative of BW25113 spontaneously obtained (see text). Kanamycin sensitive derivatives of deletion mutants were constructed using plasmid pCP20 as described.16 Plasmids ppygA is one clone of ASKA library, a set of plasmid clones containing all predicted ORFs of E. coli.17

2.2. Assay for biofilm formation

The procedure to characterize the biofilm-forming capacity of bacteria described by O‘Toole et al.18 was generally followed. A 3 μL of cells from overnight culture was inoculated in 100 μL of Luria–Bertani (LB) medium and biofilm was allowed to form in 96-well polystyrene microtiter plates (Bio Medical Equipment, Japan) at 25°C for 24 h. Growth of cell was measured by reading the absorbance (OD650) of each well using a plate reader (Molecular Device, USA). Medium was discarded and individual wells were stained with 0.1% crystal violet (CV). Subsequently, the amount of cells attached was estimated by measuring the absorbance (OD650) of CV dissolved in 0.5% SDS by the plate reader. Then, the value of biofilm was normalized according to the amount of cells. This value (CV/growth) was termed ‘relative biofilm’, and for each strain, it was indicated as the ratio of its relative biofilm to that of wild type (KR0401).

Relative biofilm(%) = \frac{CV_m/growth_m}{CV_{KR0401}/growth_{KR0401}}

2.3. Phenotype assays

Motility was observed essentially as described by Wolfe and Berg.19 Three microlitres of overnight cultures were spotted on semi-solid agar plates (1% Tryptone, 0.5% NaCl, and 0.3% Difco agar) and incubated at 30°C for 6 h. The diameter of swarming colony was measured and clones that showed more than 50% of the wild-type control as well as those that were less than 50% but apparently motile were scored as positive and intermediate phenotype, respectively. Mannose-binding Type 1 fimbriae production was examined by the ability of cells to agglutinate budding yeast cells.20 Equal volumes of bacterial and yeast cultures were mixed in a titer plate and observed for agglutination either with the naked eyes or under the microscope. Clones that showed no visible aggregation were evaluated as Type 1 fimbriae deficient. Curli fimbriae production of colonies was judged on CFA plates containing 0.1 mg/mL of Congo Red dye.21 Colonies were observed for uptake of the red colour after 3 days incubation at 25°C. Clones that were stained as much as wild-type control cells were scored as curli positive and those that remained uncoloured as deletion mutants of csgA were considered negative. Cells that showed in between colour level were evaluated as intermediate.

2.4. Preparation and analysis of OMPs

Strains were grown in 10 mL LB medium containing 30 μg/mL kanamycin to an OD600 of 0.8–1.0 and the OD600 of each culture recorded at the time of harvest. OMPs were prepared following the procedure described by Onufryk et al.22. Finally, the sample was suspended in 40 μL SDS buffer and a portion corresponding to 2.0 OD600 of cells was analyzed by loading on a 10% polyacrylamide-SDS gel.

2.5. Synthetic phenotype analysis of double deletion mutants

To examine the effect of introducing a second deletion mutation, the kanamycin resistance gene was first eliminated from deletion mutants through FLP recombination mediated by pCP20.16 The resultant KmS deletion mutants were used as recipients and another deletion mutation harbouring the antibiotic resistant cassette was introduced by P1 transduction. Appearance of KmR transductants was monitored after overnight incubation at 37°C.

3. Results and discussion

3.1. Screening of genes involved in biofilm formation

To investigate the effect of deletion of individual genes on biofilm formation, mutants were grown in LB medium and the amount of cells attached to 96-well polystyrene microtiter plates was measured. The results obtained revealed that the growth of deletion mutants was quite variable (Fig. 1), which might affect the amount of attached cells. For this reason, we normalized the value of biofilm formation against the cell growth and termed it as ‘relative biofilm’ as described in experimental procedures. Throughout these analyses, we used strain KR0401, a derivative of BW25113 (parental strain of Keio collection), as a control and the relative biofilm formation of its relative biofilm to that of wild type (KR0401).
formed by each mutant was expressed as a ratio to that of KR0401 to minimize the experimental fluctuation. KR0401 that was obtained spontaneously from BW25113 showed a stable and higher level of biofilm formation compared with BW25113 itself (data not shown). Although it is unclear why the biofilm formation of BW25113 varies from one batch to another, this feature might explain the difference in the biofilm formation observed between two deletion strains of the same gene within the Keio collection.

We analyzed the biofilm formation of each mutant at least twice and selected ~160 genes that showed, on average, less than 36% biofilm formation of KR0401 as candidates of biofilm-related gene. However, the Keio collection contains two independent sets of deletion mutants and the results for some genes were inconsistent between them. Therefore, we also selected those clones if one of the two showed less than 30% of the biofilm formation of KR0401, which is why the values of some selected clones in Fig. 1 are rather high. Next, we tried to confirm that the deletion mutations of selected genes are indeed responsible for the reduced biofilm formation by transforming with the plasmid clone of ASKA library containing each corresponding wild-type gene.23

Several deletion mutations were non-transferable by P1 phage, because strains harbouring them were refractory to P1 propagation. Therefore, we initially introduced the corresponding archive clone (ASKA library) by transformation and then propagated P1 from the resulting transformants. These genes were lpcA, rfaD, and rfaE, which are involved in LPS biosynthesis. The P1 lysates thus obtained were subsequently transduced into KR0401 and the biofilm forming ability examined. However, in the case of rfaC, tolQ, and yisS gene mutants, transformants of the corresponding archive clones still could not propagate P1 and thus we failed to confirm the effect of the deletion mutations in biofilm formation. Therefore, these genes were not included in the final list of biofilm-related genes.

As a consequence, 110 genes were identified to be associated with biofilm formation upon disruption, although there were variations in the degree of reduction observed in each gene deletion mutant (Table 1). They are classified according to their functions and their possible roles in biofilm formation are discussed in the following sections.

3.2. Motility and fimbriae genes

Various investigations reported so far showed that bacterial cell surface structures are important for cell adhesion during the development of biofilm. In *E. coli* K12 strains, flagella, Type 1, and curli fimbriae are the main structures implicated in biofilm formation.24 Therefore, we subsequently examined deletion mutants of these three surface appendages as listed in Table 1.

As expected, most mutants of flagella biosynthesis and motility genes including cydA and crp that are required for the expression of flagella genes showed severe biofilm defects and loss of motility, whereas *flhE*, *flIL*, and *flIT* mutants retained the motility as reported previously and showed a lesser extent of reduction in biofilm formation. This was also the case with an *flgN* mutant. Proteins encoded by *flgN* and *flIT* act as chaperones in flagella assembly but they are not structural proteins.28 The functions of *flhE* and *flIL* are still not clear, although they reside in the flagella operon.26,27,28 These results indicate that the biofilm formation can reflect even a subtle functional or structural difference in flagella.
Table 1. *Escherichia coli* genes of which mutation cause defective biofilm formation a

| Function | Gene | Biofilm b (%) | Phenotype c | COG | Description |
|----------|------|---------------|-------------|-----|-------------|
|          |      | av | SD | motility | Type 1 |          |              |
| Motility |      |    |   |          |        |          |              |
| flgA     | 16.1 | 7.6 | -  | +        | N, O   | Assembly protein for flagellar basal-body periplasmic P ring |
| flgB     | 17.5 | 16.4 | -  | +        | N      | Flagellar component of cell-proximal portion of basal-body rod |
| flgC     | 16.9 | 6.0 | -  | +        | N      | Flagellar component of cell-proximal portion of basal-body rod |
| flgD     | 15.4 | 10.3 | -  | +        | N      | Flagellar hook assembly protein |
| flgE     | 14.0 | 6.5 | -  | +        | N      | Flagellar hook protein |
| flgF     | 12.8 | 5.7 | -  | +        | N      | Flagellar component of cell-proximal portion of basal-body rod |
| flgG     | 20.6 | 16.4 | -  | +        | N      | Flagellar component of cell-distal portion of basal-body rod |
| flgH     | 16.8 | 5.4 | -  | +        | N      | Flagellar protein of basal-body outer-membrane L ring |
| flgI     | 20.2 | 11.9 | -  | +        | N      | Predicted flagellar basal body protein |
| flgJ     | 22.5 | 8.1 | -  | +        | N, M, O, U | Muramidase |
| flgK     | 16.4 | 5.8 | -  | +        | N      | Flagellar hook-filament junction protein 1 |
| flgL     | 10.2 | 4.4 | -  | +        | N      | Flagellar hook-filament junction protein |
| flgN     | 47.1 | 16.3 | -  | +        | N, O, U | Export chaperone for FlgK and FlgL |
| flhA     | 22.1 | 5.6 | -  | +        | N      | Predicted flagellar export pore protein |
| flhB     | 16.4 | 5.0 | -  | +        | N, U   | Predicted flagellar export pore protein |
| flhC     | 17.7 | 3.5 | -  | +        |        | DNA-binding transcriptional regulator with FlhD |
| flhD     | 28.7 | 4.4 | -  | +        |        | DNA-binding transcriptional dual regulator with FlhC |
| flhE     | 56.1 | 27.7 | v  | +        |        | Flagellar protein |
| fliA     | 18.9 | 6.9 | -  | +        |        | RNA polymerase, sigma 28 (sigma F) factor |
| fliC     | 18.1 | 5.8 | -  | +        | N      | Flagellar filament structural protein (flagellin) |
| fliD     | 13.4 | 5.8 | -  | +        | N      | Flagellar filament capping protein |
| fliEa    | 13.8 | 5.7 | -  | +        | N, U   | Flagellar basal-body component |
| fliF     | 19.1 | 8.1 | -  | +        | N, U   | Flagellar basal-body MS-ring and collar protein |
| fliG     | 14.4 | 2.3 | -  | +        | N      | Flagellar motor switching and energizing component |
| fliH     | 13.6 | 8.7 | -  | +        | N, U   | Flagellar biosynthesis protein |
| fliI     | 18.7 | 3.2 | -  | +        | N, U   | Flagellum-specific ATP synthase |
| fliJ     | 10.9 | 4.4 | -  | +        | N, O, U | Flagellar protein |
| fliK     | 19.1 | 4.1 | -  | +        | N      | Flagellar hook-length control protein |
| fliL     | 46.6 | 4.4 | +  | +        | N      | Flagellar biosynthesis protein |
| fliM     | 21.9 | 9.4 | -  | +        | N      | Flagellar motor switching and energizing component |
| fliN     | 18.7 | 4.9 | -  | +        | N, U   | Flagellar motor switching and energizing component |
| fliO     | 9.5  | 3.8 | -  | +        |        | Flagellar biosynthesis protein |
| fliP     | 18.1 | 6.0 | -  | +        | N, U   | Flagellar biosynthesis protein |
| fliQ     | 17.0 | 3.7 | -  | +        | N, U   | Flagellar biosynthesis protein |
| fliR     | 15.3 | 5.2 | -  | +        | N, U   | Flagellar export pore protein |
| fliS     | 26.8 | 6.0 | -  | +        | N, O, U | Flagellar protein potentiatess polymerization |
| fliT     | 59.0 | 4.9 | +  | +        |        | Predicted chaperone |
| motA     | 52.5 | 14.5 | -  | +        | N      | Proton conductor component of flagella motor |
| motB     | 15.9 | 4.4 | -  | +        | N      | Protein that enables flagellar motor rotation |
| Type 1   |      |    |   |          |        |          |              |
| fimA     | 3.9  | 3.9 | +  | -        | N, U   | Major Type 1 subunit fimbrin (pilin) |
| fimB     | 1.2  | 1.4 | +  | -        | L      | Tyrosine recombinase/inversion of on/off regulator of fimA |

Continued
| Function | Gene   | Biofilm (%) | Phenotype | COG       | Description                                      |
|----------|--------|-------------|-----------|-----------|--------------------------------------------------|
|          |        | av SD       | motility  | Type 1    | curli                                            |
|          |        |             |           |           |                                                  |
|          | fimC   | 1.0 1.3     | +         | –         | + N, U Chaperone, periplasmic                      |
|          | fimD   | 1.4 1.9     | +         | –         | + L Outer membrane usher protein, Type 1 fimbrial synthesis |
|          | fimF   | 1.2 1.8     | +         | –         | + N, U Minor component of Type 1 fimbriae        |
|          | fimG   | 17.5 15.1   | +         | +         | + N, U Minor component of Type 1 fimbriae        |
|          | fimH   | 1.1 1.5     | +         | –         | + minor component of Type 1 fimbriae             |
| Curli    | csgA   | 32.9 4.8    | +         | +         | – Cryptic curlin major subunit                    |
|          | csgB   | 33.1 3.3    | +         | +         | – Curlin nucleator protein, minor subunit in curli complex |
|          | csgC   | 52.9 1.6    | +         | +         | – DNA-binding transcriptional activator in two-component regulatory system |
|          | csgE   | 43.8 7.6    | –         | –         | – Predicted transport protein                     |
|          | csgF   | 45.2 8.4    | +         | +         | + Predicted transport protein                     |
|          | csgG   | 12.5 2.0    | –         | +         | + Outer membrane lipoprotein                     |
| LPS      | lpcA   | 9.6 1.0     | –         | +         | + G D-sedoheptulose 7-phosphate isomerase         |
|          | gmbhB  | 24.7 4.1    | +         | +         | + E, G, M D, D-heptose 1,7-bisphosphate phosphatase |
|          | rfaD   | 12.5 2.0    | –         | +         | + ADP-L-glycero-D-mannoheptose-6-epimerase, NAD(P)-binding |
|          | rfaE   | 11.4 2.3    | –         | +         | + M Fused heptose 7-phosphate kinase/heptose 1-phosphate adenyltransferase |
|          | rfaF   | 13.2 2.2    | +         | +         | + M ADP-heptose:LPS heptosyltransferase II         |
|          | rfaG   | 19.1 9.9    | +         | +         | + M Glucosyltransferase I                         |
|          | rfaH   | 22.7 13.7   | +         | +         | + K DNA-binding transcriptional antiterminator   |
|          | rfaP   | 12.3 6.4    | +         | +         | + Kinase that phosphorylates core heptose of lipopolysaccharide |
| Other    | btuB   | 72.5 27.2   | +         | v         | + H Vitamin B12/cobalamin outer membrane transporter |
|          | choZ   | 65.4 15.4   | –         | +         | + N, T Chemotaxis regulator                      |
|          | crp    | 8.9 4.5     | –         | +         | + T DNA-binding transcriptional dual regulator   |
|          | crr    | 22.5 10.6   | +         | +         | + G Glucose-specific enzyme IIA component of PTS |
|          | cyaA   | 4.5 4.3     | –         | +         | – F Adenylate cyclase                            |
|          | degP   | 38.4 7.4    | +         | +         | + O Serine endopeptase (protease Do), membrane-associated |
|          | dkgA   | 76.7 6.3    | +         | +         | + M Diacylglycerol kinase                        |
|          | dnaK   | 46.2 5.8    | –         | +         | + O Chaperone Hsp70, co-chaperone with DnaJ       |
|          | dsbA   | 8.5 3.3     | +         | +         | + C,O Periplasmic protein disulfide isomerase I   |
|          | dsbB   | 43.6 11.5   | +         | +         | + Oxidoreductase that catalyzes reoxidation of DsbA protein disulfide isomerase I   |
|          | fruR   | 52.3 3.0    | +         | +         | + K DNA-binding transcriptional dual regulator   |
|          | galU   | 24.3 11.7   | +         | +         | + M Glucose-1-phosphate uridylyltransferase       |
|          | gcvA   | 74.6 16.9   | +         | +         | + K DNA-binding transcriptional dual regulator   |
|          | greA   | 49.8 3.5    | +         | +         | + K DNA-binding transcriptional dual regulator   |
|          | hfg    | 43.6 4.1    | +         | +         | + R HF-I, host factor for RNA phage Q beta replication |
|          | hscB   | 68.0 14.6   | +         | +         | + O DnaJ-like molecular chaperone specific for IscU |
|          | hsrA   | 70.8 3.7    | +         | +         | – E, G, P, R Predicted multidrug or homocysteine efflux system |
|          | gspO   | 2.2 2.0     | +         | –         | – L Integration host factor (IHF), DNA-binding protein, beta subunit |
|          | lon    | 37.3 4.3    | +         | +         | – O DNA-binding ATP-dependent protease La         |
|          | mdoH   | 60.1 10.4   | +         | +         | + M Glucan biosynthesis: glyosyl transferase     |

Continued
Table 1. Continued

| Function | Gene       | Biofilmb (%) | Phenotypec | COG        | Description                                          |
|----------|------------|--------------|------------|------------|------------------------------------------------------|
|          |            | av  SD       | motility   | Type 1     | curli                                               |
| av       | 61.5       | 4.9          | +          | +          | − K DNA-binding transcriptional regulator            |
| mlrA     | 64.1       | 5.1          | +          | +          | + H Predicted molybdochelatase                       |
| mltE     | 72.0       | 12.6         | +          | +          | + G N-acetylglucosamine-6-phosphate deacetylase      |
| mgtA     | 56.8       | 4.6          | +          | +          | + R Conserved protein                                |
| nagA     | 68.3       | 12.6         | +          | +          | + N, U Membrane spanning protein in TolA-TolQ-TolR complex |
| yjha/    | 43.5       | 3.8          | +          | +          | + S Conserved protein                                |
| nanC     | 58.6       | 4.1          | +          | +          | + G Succinate dehydrogenase, membrane subunit, binds cytochrome b556 |
| ndfU     | 60.0       | 4.2          | +          | +          | + N, U Periplasmic protein                           |
| npdI     | 56.8       | 4.6          | +          | +          | + R Conserved protein                                |
| ompR     | 47.8       | 3.7          | +          | +          | + N, U Membrane spanning protein in TolA-TolQ-TolR complex |
| pgi      | 59.2       | 4.0          | +          | +          | + G Glucosephosphate isomerase                       |
| proQ     | 66.6       | 10.4         | +          | +          | + G Succinate dehydrogenase, membrane subunit, binds cytochrome b556 |
| ptsI     | 63.4       | 9.3          | +          | +          | + G PEP-protein phosphotransferase of PTS system (enzyme I) |
| rcsC     | 72.9       | 3.9          | +          | +          | + T Hybrid sensory kinase in two-component regulatory system with RcsB and YojN |
| rpmE     | 58.2       | 4.1          | +          | +          | + M Membrane anchored protein in TolA-TolQ-TolR complex |
| rpmS     | 58.6       | 4.2          | +          | +          | + M Membrane anchored protein in TolA-TolQ-TolR complex |
| sdhC     | 73.3       | 3.6          | +          | +          | + C Succinate dehydrogenase, membrane subunit, binds cytochrome b556 |
| surA     | 3.3        | 2.9          | +          | −          | + O Peptidyl-prolyl cis–trans isomerase (PPIase)     |
| tolA     | 58.6       | 4.1          | +          | +          | + M Membrane anchored protein in TolA-TolQ-TolR complex |
| tolB     | 45.6       | 4.8          | +          | +          | + N, U Periplasmic protein                           |
| tolR     | 45.9       | 2.3          | +          | +          | + N, U Membrane spanning protein in TolA-TolQ-TolR complex |
| yfgL     | 37.5       | 10.5         | +          | +          | + S Protein assembly complex, lipoprotein component |
| yefM     | 50.4       | 7.3          | ±          | +          | + N, R Predicted outer membrane lipoprotein          |
| yciB/    | 48.0       | 12.2         | ±          | +          | + D Predicted inner membrane protein                  |
| ispZ     | 39.9       | 14.0         | ±          | +          | + D Predicted inner membrane protein                  |
| ycdM     | 58.2       | 4.3          | ±          | +          | + T Predicted diguanylate cyclase, GGDEF domain signalling protein |
| ydeT/    | 60.5       | 18.1         | +          | +          | + N, U Predicted protein                             |
| (finD)   | 63.3       | 15.1         | −          | +          | + S Conserved protein                                |
| yfgA     | 68.3       | 19.4         | +          | +          | + S Conserved protein                                |
| yhcB     | 75.7       | 39.0         | +          | +          | + R Predicted xanthine/uracil permease               |
| yncC     | 41.0       | 7.1          | +          | +          | + S Conserved protein                                |

Genes are classified according to their known function. Name, clusters of orthologous group (COG), and description of genes are adapted from GenoBase (http://ecoli.naist.jp). An alternative gene name is given in addition to the systematic name when available. Average (av) and standard deviation (SD) of relative biofilm formation were calculated and normalized to the values of KR0401 with more than four transductants for each deletion mutation. Phenotype of motility, Type 1, and curli fimbriae were examined as described in experimental procedures. +, −, ±, and v indicate normal, defective, intermediate, and variable among transductants, respectively.
This possibility was also indicated in the results for mutants of disulfide interchange proteins. A severe biofilm defect (8.5% of wild type) was observed with a dsbA mutant, whereas dsbB disruption caused only a mild reduction (50%). DsbA protein catalyzes the disulfide bond formation, whereas the role of DsbB is to supply the material and its requirement can be suppressed in a medium supplied with cysteine.30 Dsb proteins are implicated in flagella assembly and mutants are non-motile in the absence of cysteine.31 Flagella are probably important for the initial cell-to-surface contact and the spread of bacteria along the surface.10 Previously we reported that the overproduction of several genes for flagella biosynthesis also showed reduced biofilm formation.23 This reduced biofilm phenotype might have resulted from the uncoordinated gene expression that has led to the deficiency of flagella and reduced motility.

Apart from flagella-related genes, mutants of lpcA, rfaD, and rfaE that are required for LPS biosynthesis were found to be non-motile and highly impaired in biofilm formation. On the other hand, cheZ, dnaK, and yfgA mutants are non-motile and yet showed only moderate biofilm phenotypes. These observations indicate that the structure of flagella and membrane that supports the biofilm phenotypes. These observations indicate that the structure of flagella and membrane that supports the biofilm formation. The results mentioned above not only confirmed the importance of these cell surface structures but also indicated that the function of a specific deletion was well reflected in the biofilm formation. On the other hand, the existence of many more mutants that seem to be intact in these surface structures but exhibit reduced biofilm phenotype showed that indeed various genes are required for the proper development of biofilm.

3.3. Lipopolysaccharide genes

Genome-wide analysis of deletion mutants revealed that eight genes involved in lipopolysaccharide (LPS) synthesis exhibited a significant degree of reduction in biofilm formation when disrupted. All of them except rfaH encode enzymes that catalyze the synthesis of L-glycerol-3-phosphate and inner core assembly of the LPS indicating that the heptose region of the core oligosaccharide is important not only for the outer membrane stability37 but also for the adhesion of cell whereas the outer core and O-antigen are not critical for biofilm formation. Their defective biofilm formation seems not simply due to the lack of the major surface structures mentioned above, at least in the case of rfaF, rfaG, rfaH, and rfaP mutants, because they were normal in motility and agglutination assay. LPS seems to be important for the initial attachment to the surface, since mutants of LPS synthesis genes showed biofilm reduction similar to flagella-defective mutants. Some of them also exhibited motility-defective phenotype as described earlier. The gene rfaH encodes a transcriptional antiterminator required for the expression of the rfa operon38 and, in contrast to our result, its inactivation was reported to increase the initial adhesion and biofilm formation.39 The reason for this discrepancy is not clear but it might be due to the difference in culture conditions.
3.4. Others

Apart from LPS, many genes related to cell membrane were identified to exhibit mild reduction in biofilm formation upon disruption. However, our analysis could not identify the genes for colanic acid synthesis. This exopolysaccharide is not required for the initial attachment but important for the biofilm development into a complex three-dimensional structure (reviewed by Van Houdt and Michiels24). Therefore, the possible reason for this failure is that our procedure was not suitable for the analysis of the late stage of biofilm formation or that the parental strain of the Keio collection does not develop such a structure as reported for some laboratory E. coli strains.

In addition, genes of more variable functions, including nine genes of unknown function, were identified in our analysis. Although the precise mechanism how the deletion of these genes leads to defective biofilm formation remains to be clarified, it is conceivable that the perturbation of cellular activity such as metabolism and energy production causes some deficiency or stress in the cell membrane, thereby affecting biofilm formation.

3.5. Characterization of function-unknown genes

Some of the function-unknown genes identified are predicted to encode membrane proteins based on their primary structure and/or the phenotypes of their mutants (Table 1). Moreover, it is well expected that membrane proteins, in particular OMPs, contribute to the developmental processes of biofilm formation. Therefore, we examined the profiles of major OMPs isolated from mutants of function-unknown genes and surA. SurA possesses both periplasmic chaperone and peptidyl prolyl isomerase (PPIase) activities and facilitates OMP biogenesis.40 Its mutant shows a profile of significantly reduced OMPs41 and highly impaired biofilm formation (Table 1). The amounts of OmpA, OmpC, OmpF, and LamB were found to be reduced in yciB, yciM, and yfgA mutants, although the effect was not so drastic as that observed with the surA mutant (Fig. 2). This result indicates that these genes might be required for membrane integrity. However, our analysis did not identify mutants of major OMP genes: the reason could be that deficiency of a single OMP gene might not cause an observable reduction in biofilm formation.

The genetic interaction of function-unknown genes with the surA gene was then investigated by analyzing the synthetic phenotype of double mutations as described in the experimental procedures. By observing the phenotype of the double mutants thus constructed, ΔyfgA was found to exhibit a synthetic lethal phenotype with ΔsurA. Similarly, ΔyciB, ΔyciM, and ΔyhcB were synthetically lethal with ΔyfgA and the phenotype was rescued by introducing a plasmid carrying the wild-type allele of yfgA (Table 2). Mutants of yciM and yfgA were also more sensitive to SDS and Novobiocin, an amphipathic antibiotic, compared with the wild-type strain (data not shown).

Table 2. Synthetic phenotypes displayed by function-unknown genes

| P1 donors | ΔsurA | ΔyfgA | ΔyfgA(pyfgA) |
|-----------|-------|-------|--------------|
| ΔyciB::Km | +     | vs/−  | +            |
| ΔyciM::Km | vs    | −     | −            |
| ΔyfgA::Km | −     | NT    | NT           |
| ΔyhcB::Km | +     | −     | +            |

+ indicates much the same phenotype as that of the corresponding single mutant, whereas − indicates that no transductant appeared, and vs very small colonies, respectively. NT, not tested.

Figure 2. The OMP profiles of deletion mutants of uncharacterized genes. Outer membrane fractions prepared from the equivalent amount of cells of KR0401 (lane 1) and its derivative strains harboring ΔyfgA::Km, ΔyciM::Km, ΔyciB::Km, and ΔsurA::Km mutations (lanes 2–5, respectively) were analyzed on a 10 % SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue.

3.6. Concluding remarks

Our genome-wide analysis demonstrated that variable genes are indeed required for biofilm formation and that the cell surface structures and envelope are important factors. It could be that cells are required to re-organize their membrane structures during the process of developing biofilms and it is in such a process that the hitherto uncharacterized genes described above might be involved.

The analysis described here by using deletion mutants could directly identify genes involved in biofilm formation. However, certain genes not identified in this analysis might probably be required under different conditions and/or in different genetic backgrounds. Also, we found genes that increased biofilm formation upon disruption (Fig. 1). They include various genes such as those for signal transduction, transcription, carbohydrate metabolism as well as those of unknown function.
Further analysis of these genes would provide clues with respect to how bacteria change their life-style.

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