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ENVIRONMENTAL BIOTECHNOLOGY

Establishment of a multi-species biofilm model and metatranscriptomic analysis of biofilm and planktonic cell communities

Yuya Nakamura[1] · Nao Yamamoto[1] · Yuta Kino[1] · Nozomi Yamamoto[2] · Shota Kamei[1] · Hiroshi Mori[1] · Ken Kurokawa[1,2] · Nobutaka Nakashima[1]

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Abstract We collected several biofilm samples from Japanese rivers and established a reproducible multi-species biofilm model that can be analyzed in laboratories. Bacterial abundance at the generic level was highly similar between the planktonic and biofilm communities, whereas comparative metatranscriptomic analysis revealed many upregulated and downregulated genes in the biofilm. Many genes involved in iron-sulfur metabolism, stress response, and cell envelope function were upregulated; biofilm formation is mediated by an iron-dependent signaling mechanism and the signal is relayed to stress-responsive and cell envelope function genes. Flagella-related gene expression was regulated depending upon the growth phase, indicating different roles of flagella during the adherence, maturation, and dispersal steps of biofilm formation. Downregulation of DNA repair genes was observed, indicating that spontaneous mutation frequency would be elevated within the biofilm and that the biofilm is a cradle for generating novel genetic traits. Although the significance remains unclear, genes for rRNA methyltransferase, chromosome partitioning, aminoacyl-tRNA synthase, and cysteine, methionine, leucine, thiamine, nucleotide, and fatty acid metabolism were found to be differentially regulated. These results indicate that planktonic and biofilm communities are in different dynamic states. Studies on biofilm and sessile cells, which have received less attention, are important for understanding microbial ecology and for designing tailor-made anti-biofilm drugs.

Keywords Biofilm · DNA repair · Iron metabolism · Metatranscriptome · Planktonic cell · RNA-seq · Stress response · Sulfur metabolism

Introduction

Natural environments and multicellular organisms are colonized by various microbes. In many cases, microbial cells form complex communities wherein they communicate (e.g., quorum sensing signals), cooperate (e.g., symbiosis of *Clostridium acetobutylicum* and *Bacillus cereus*; Wang et al. 2015), and fight (e.g., production of antimicrobials) each other, instead of existing as isolated cells. Recent studies propose that microbial communities are mostly present in a sessile state and not in a planktonic state (Watnick and Kolter 2000). Since microbes are usually cultured as free planktonic cells in laboratory experiments, the sessile state needs to be given more attention in order to understand microbial activities.

A biofilm is a population of cells growing on a surface and is a type of sessile cell community. Biofilm structures are ubiquitously found on both abiotic and biotic surfaces like kitchen sinks, river stones, and human teeth. Biofilms are known to be responsible for nosocomial infections, contamination of medical devices, chronic bacterial infections, and periodontal diseases (Donlan and Costerton 2002). Microbial cells within biofilm structures are packaged along with extracellular polysaccharides (EPS), nucleic acids, proteins, and other materials, and are tolerant to antimicrobials,

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✉ Nobutaka Nakashima
n-nakashima@aist.go.jp

[1] Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 2-12-1-M6-5 Ookayama, Meguro-ku, Tokyo 152-8550, Japan

[2] Earth-Life Science Institute, Tokyo Institute of Technology, 2-12-1-M6-5 Ookayama, Meguro-ku, Tokyo 152-8550, Japan

heat treatment, and the host immune system (Carvalhais et al. 2015; Stoodley et al. 2002). Therefore, it is difficult to kill microbial cells inside a biofilm and to eliminate the entire biofilm structures. As an example, in the lungs of patients with cystic fibrosis (a genetic disorder), pathogenic *Pseudomonas aeruginosa* cells form strong biofilms and acquire high tolerance to medical treatments (Magalhães et al. 2016).

Biofilm research so far has mainly focused on biofilm samples consisting of one or a few microbial species, but only a limited number of studies have been performed that concern complex multi-species biofilms. It is therefore important to perform meta 16S ribosomal RNA (rRNA) gene and metatranscriptome analyses of biofilm samples from complex microbiomes or environments. These analyses would be helpful for a detailed understanding of microbial ecology and the development of anti-biofilm drugs.

In the present study, we cultured biofilm samples collected from Japanese rivers and succeeded in establishing a multi-species biofilm model in the laboratory. Culture of this biofilm model resulted in the formation of both planktonic and biofilm cell communities. Although meta 16S rRNA analysis showed little difference between the planktonic and biofilm cell communities, metatranscriptomic analysis revealed various differentially expressed genes.

Materials and methods

Sample collection and biofilm culture

Biofilm samples were cultured in one third strength of Luria broth (1/3 LB; LB broth was purchased from Difco, Sparks, MD, USA) at 25 °C, unless otherwise stated.

Ten river samples were collected from different points of the Tamagawa River, including a rock covered with biofilm from midstream (north latitude 35.610566 and east longitude 139.624680), a stone and its covering slime from midstream (35.610461, 139.625142), a stone and covering slime from downstream (35.541471, 139.752706), brown slime from downstream (35.541471, 139.752706), slime attached to the underside of a rock from downstream (35.541471, 139.752706), slime attached to the underside of a mat from downstream (35.541471, 139.752706), a stone and covering slime from upper stream (35.621639, 139.572920), slime of the top of driftwood from upper stream (35.621642, 139.572930), river water from upstream (35.621700, 139.572478), and river water from downstream (35.541471, 139.752706). Three samples were collected from the Yazawagawa River: river water (35.6037972, 139.6464361), waterfall (35.6037972, 139.6464361), and red clay (35.605759, 139.645380). Since this location is not privately owned or protected, no specific permits were required for the described field studies. A sterilized slide glass was placed in a Petri dish (90 mm diameter), and 10 ml of 1/3 LB along with a drop of the sample (mixture of biofilm and river water) was added. Three-day-old statically cultured cells (both planktonic and biofilm cells) were used to prepare glycerol stocks of these samples with 20 % glycerol and stored at −80 °C.

After a week, inoculation was initiated from glycerol stocks in a similar manner using 50 μl of the glycerol stock solution from each sample. After 3 days, planktonic and biofilm cells were collected separately. (i) The planktonic cells were collected by aspirating 1.5 ml culture broth, (ii) the remaining culture broth was discarded, (iii) the inner part of the dish and the surface of the slide glass were washed twice with 5 ml of 1× PBS [made using 10× PBS(−) purchased from Wako Pure Chemical Industries, Osaka, Japan], (iv) 800 μl of 1× PBS was added, and (v) the biofilm cells were scraped off from the inner part of the dish and the surface of the slide glass using an interdental brush (Yawaraka-Shikan-Burashi; Kobayashi Seiyaku, Tokyo, Japan). Glycerol stocks were prepared again as described above, and these culture and sample collection steps were repeated two more times (thrice in total).

DNA extraction and meta 16S rRNA sequence analysis

Metagenomic DNA from the original biofilm samples and the cultured planktonic and biofilm cells was prepared using a Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), according to the manufacturer's instructions. The V3–V4 regions of the 16S rRNA genes (position 342 to 806 in *Escherichia coli* numbering) were PCR-amplified using a non-degenerate universal primer set, 342F and 806R (Kato et al. 2015), with EX Taq HS polymerase (Takara Bio, Ohtsu, Japan). All PCR samples were mixed to generate a 21-plex sample library, and the sequences were read by the MiSeq instrument (Illumina, San Diego, CA) in a paired-end-read mode for 300 cycles according to the manufacturer's protocol.

High-quality sequences (of >150 and <450 bases in length) were selected for subsequent processing (i.e., alignment to a standard sense strand and removal of the primer sequences used for sequencing). Only the forward sequences were used for subsequent processing since the reverse sequences were mostly of low quality and merging of each forward and reverse sequence was not possible. Sequence clustering of these high-quality reads was performed using the UCLUST program (Edgar 2010) version 6.0.307 with an identity of ≥97 % and a query and reference coverage of ≥80 %. Chimeric clusters detected by the UCHIME program (Edgar et al. 2011) in the de novo mode and the reference mode [searching the reference Gold Database (DB) (http://drive5.com/uchime/gold.fa)] were removed. Taxonomic assignment of the resulting MiSeq reads was performed using the RDP Classifier program (Wang et al. 2007) version 2.6 with a bootstrap value ≥0.5 against the representative sequences of each

97 % sequence cluster chosen by the UCLUST program. Unless otherwise stated, the programs for read assemblage and analysis in this study were run using default parameters. The 16S rRNA sequence data have been deposited under the BioProject accession number PRJDB4599 in the DDBJ BioProject database (http://trace.ddbj.nig.ac.jp/bioproject/index_e.html).

mRNA extraction and construction of a cDNA library

The culture conditions and sample collection methods were the same as described above. The culture was started with the second glycerol stock of the sample, and 30 Petri dishes were handled in parallel to obtain sufficient amounts of RNA for RNA-seq. Cells were collected separately from the planktonic and the biofilm communities from 12, 24, and 48-h cultures. Total RNA was extracted from the cultured biofilm and planktonic cells using the acid hot phenol method (Aiba et al. 1981). The extracted RNA samples were purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for enriching the messenger RNA (mRNA), since most RNAs of <200 nucleotides (including 5.8S rRNA, 5S rRNA, and transfer RNAs (tRNAs), together constituting 15–20 % of total RNA) are selectively excluded. rRNAs were removed from total RNA using a MICROBExpres Bacterial mRNA Enrichment Kit (Ambion, Austin, TX). Complementary DNA (cDNA) libraries were then constructed using a TruSeq Stranded mRNA and Total RNA sample preparation kit (Illumina) and sequenced using MiSeq in the paired-end-read mode for 300 cycles.

RNA-seq analysis

High-quality RNA-seq reads (of >150 and <450 bases in length) were selected for subsequent processing (i.e., alignment to a standard sense strand and removal of primer sequences used for sequencing). RNA-seq reads from each sample were separately assembled using the IDBA-UD program (Peng et al. 2012) version 1.1.0 with the parameters "–mink 20 –maxk 70 –step 5." Protein coding sequences in the contigs were predicted using the MetaGeneMark program (Zhu et al. 2010) version 2.10 with the parameters "-g 11 -a -d -f G." Functional annotation of each read was performed by BLASTP search using the parameters "-g T -F F -e 1e-8 -m 8 -b 10 -v 10" against the KEGG protein sequence DB, which was obtained in March 2014. Taxonomic (phylum, class, order, family, and genus levels) and functional [KEGG Orthology (KO)-level] assignment was performed for reads that exhibited the highest BLAST scoring hits with an identity ≥70 % and a BLAST bit-score ≥40. Reads from each sample were mapped to the predicted ORFs using Bowtie2 (Langmead and Salzberg 2012). KO abundance was calculated by dividing the number of reads belonging to the same KO by the median length of proteins annotated to the same KO in the KEGG protein DB. KO abundance was normalized to the number of reads included in each sample. KEGG pathway abundance was calculated as the sum of KO abundances belonging to a single pathway. To compare the abundances of individual KOs and their pathways among different samples, abundances in each RNA-seq sample were normalized to sample read numbers. This method for normalizing functional abundances among samples was applied to all BLAST-based functional assignments in this study. RNA-seq data have been deposited under the BioProject accession number PRJDB4600 in the DDBJ BioProject database (http://trace.ddbj.nig.ac.jp/bioproject/index_e.html).

Determination of biofilm amounts

Samples from glycerol stocks were cultured in a Petri dish (60-mm diameter) containing 2 ml medium. Only planktonic cell communities were collected from the grown samples, and their optical density at 595 nm was measured. To measure the amount of biofilm, the planktonic cells were completely removed, and the remaining biofilm samples were gently washed twice with 2 ml distilled water. The biofilm samples were kept in a fume hood with a blower until the samples dried out (approximately 1 h). Then, 2 ml of 0.1 % crystal violet solution was added to each sample and incubated for 15 min at room temperature. After discarding the crystal violet solution, the biofilm samples were washed twice with 2 ml of distilled water, gently to keep the biofilm intact. The stained biofilm samples were then dried out as previously described, in a fume hood (for approximately 1 h). To each sample, 2 ml of 70 % ethanol was added and the samples were kept for 30 min at room temperature. Finally, 100 μl of the stained ethanol was collected from each sample, and the amounts of the eluted dye were quantified by measuring absorbance at 595 nm.

Results

Establishment of a model biofilm system in a laboratory

Considering that most natural biofilms consist of multiple bacterial species, we believe that the lack of model biofilm systems has hampered intensive research in this area. To establish a biofilm model, 13 river samples were collected, including biofilm structures from both abiotic surfaces and the surrounding river water. These samples were cultured in a Petri dish containing a slide glass (see "Materials and Methods" and Fig. 1a). Three of the samples, SM (a stone and its covering slime from midstream), BD (brown slime from downstream), and WU (river water from upstream), were found to form a clear biofilm on the wall of the dish and on the slide glass after 3 days. Additionally, these three cultures could form a biofilm even when the first cultures were frozen as glycerol stocks and the

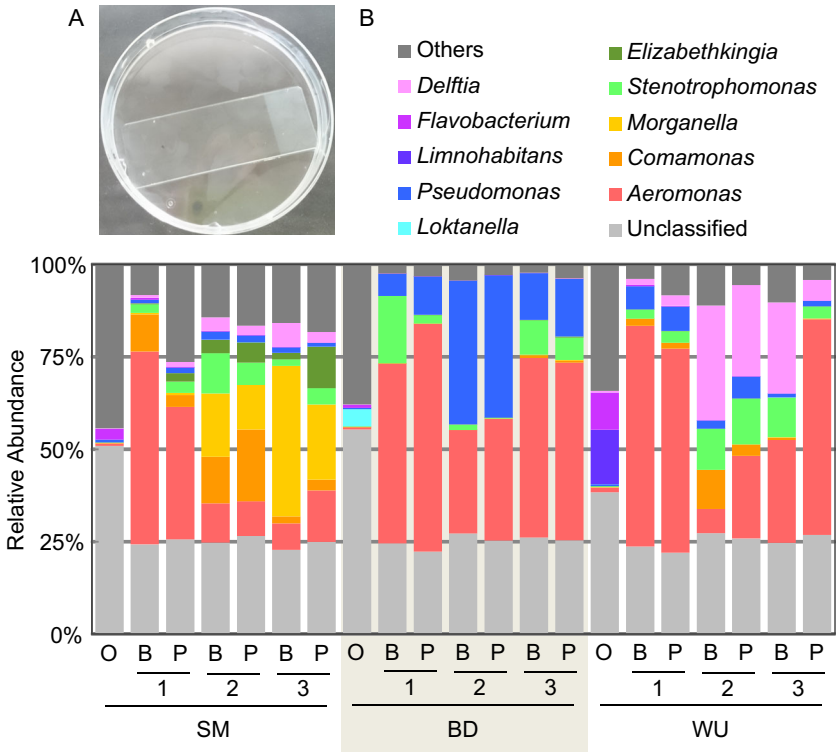

Fig. 1 Biofilm model and 16S rRNA gene analysis. **a** An image of the cultured biofilm BD is shown. **b** 16S rRNA gene sequences were classified up to the generic level. "O," "B," and "P" characters indicate original culture, biofilm community, and planktonic community, respectively. The *numerals* indicate the number of inoculations repeated

second inoculation was restarted from these stocks. In the same manner, we confirmed that biofilm formation could be repeated at least two more times.

The bacterial species existing in planktonic and biofilm communities of the above samples were analyzed by massive sequencing of 16S rRNA (Fig. 1b). The sequencing results indicate that the generic composition differed depending on the source of the biofilm and that the composition between the planktonic and biofilm communities from the same culture was not clearly different. The generic composition of the original samples (i.e., uncultured samples, Fig. 1b) was also analyzed in a similar manner. We discovered that the variation in the biofilm community was lost upon culture in laboratory conditions. Taken together, we successfully established model biofilm systems, which are reculturable and reproducible in the laboratory. Nevertheless, the fact that species composition might be variable among repeated cultures should be considered.

An overview of the RNA-seq analysis of planktonic and biofilm communities

As mentioned, phylogenetic analysis based on 16S rRNA sequences showed little difference between the planktonic and biofilm communities. In order to understand the dynamic but not the static differences among the communities, metatranscriptome analysis by RNA-seq was performed using 12-, 24-, and 48-h cultures (see "Materials and Methods"). Among the above-mentioned three samples, only the sample

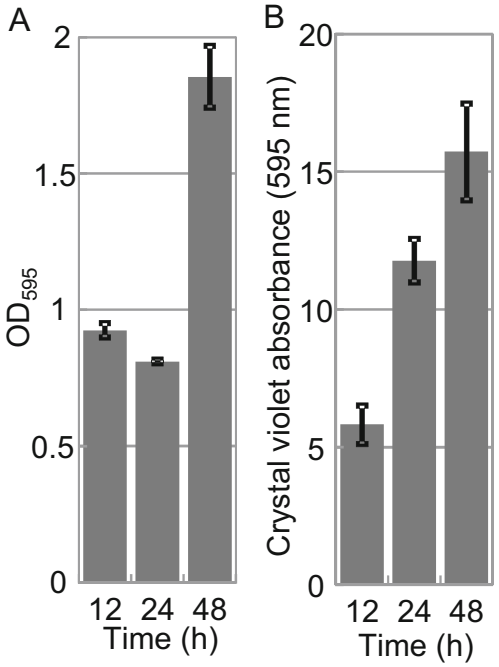

Fig. 2 Growth kinetics and quantitation of biofilm formation. **a** Growth kinetics of the BD culture are shown. Optical density at 595 nm was measured at three time points. The data are presented as mean ± SD from triplicate experiments. **b** Quantitation of crystal violet staining of the BD culture is shown. The amounts of crystal violet indicating the amounts of biofilm formed were measured at three time points. The data are presented as mean ± SD from triplicate experiments

BD formed sufficient amounts of biofilm to be subjected to RNA-seq, and therefore, only this sample was used further. The growth and biofilm formation kinetics observed during culture are shown in Fig. 2a, b, and we defined 12-, 24-, and 48-h cultures as early, middle, and late stages of biofilm formation, respectively. Growth was found to drift into a period of stagnation around the middle stage of the culture, possibly due to the co-existence of fast and slow growers in this culture.

From the metatranscriptome data, we first examined the bacterial genera and species that were alive and vital at the specific biofilm formation stages in both communities. To this end, the average expression levels of *gyrB*, *rpoB*, and *rplC* genes were determined (Fig. 3a). These three genes were chosen as a representative of the replication, transcription, and translation machineries and as universal single copy genes (Kato et al. 2015). At the early stage of culture, *Aeromonas* and *Pseudomonas* bacteria were dominant, and with time, *Pseudomonas* bacteria became highly dominant (Fig. 3a). This result is consistent with that shown in Fig. 1b. However, bacteria that were not significantly detected in meta 16S rRNA analysis were also found to be present and vital; these included (i) *Acinetobacter* at the early and middle stages, (ii) *Laribacter* and *Xanthomonas* at the middle and late stages, (iii) *Enterobacter* at the early stage of the planktonic community, and (iv) *Pseudogulbenkiania* at the late stage of the biofilm community (Fig. 3a). Although determination of bacterial species was difficult using meta 16S rRNA analysis (Fig. 1b), it was possible with the metatranscriptome data (Fig. 3b). At the species level, at least 20 bacterial species were found to be present and vital, and our culture conditions were feasible for these bacteria. It should be noted that the metatranscriptome data do not reflect the simple presence ratio of bacterial species.

In the next level of metatranscriptome data analysis, the upregulated genes in the biofilm community were identified (Table 1). Over 30 % of the genes were upregulated in the subcategories of "biosynthesis of other secondary metabolites, " "xenobiotics biodegradation and metabolism," "membrane transport," and "lipid metabolism" (Table 1, limited to subcategories in which at least 10 genes were detected). These results suggest that changes in cell envelope structure and in material import/export across the cell envelope are important for biofilm formation. In Tables 2, 3, 4, and 5, upregulated genes in the biofilm community at three or two time points are listed, and in Tables S1–S3, the genes upregulated at only one time point are listed. Notably, the genes for cysteine

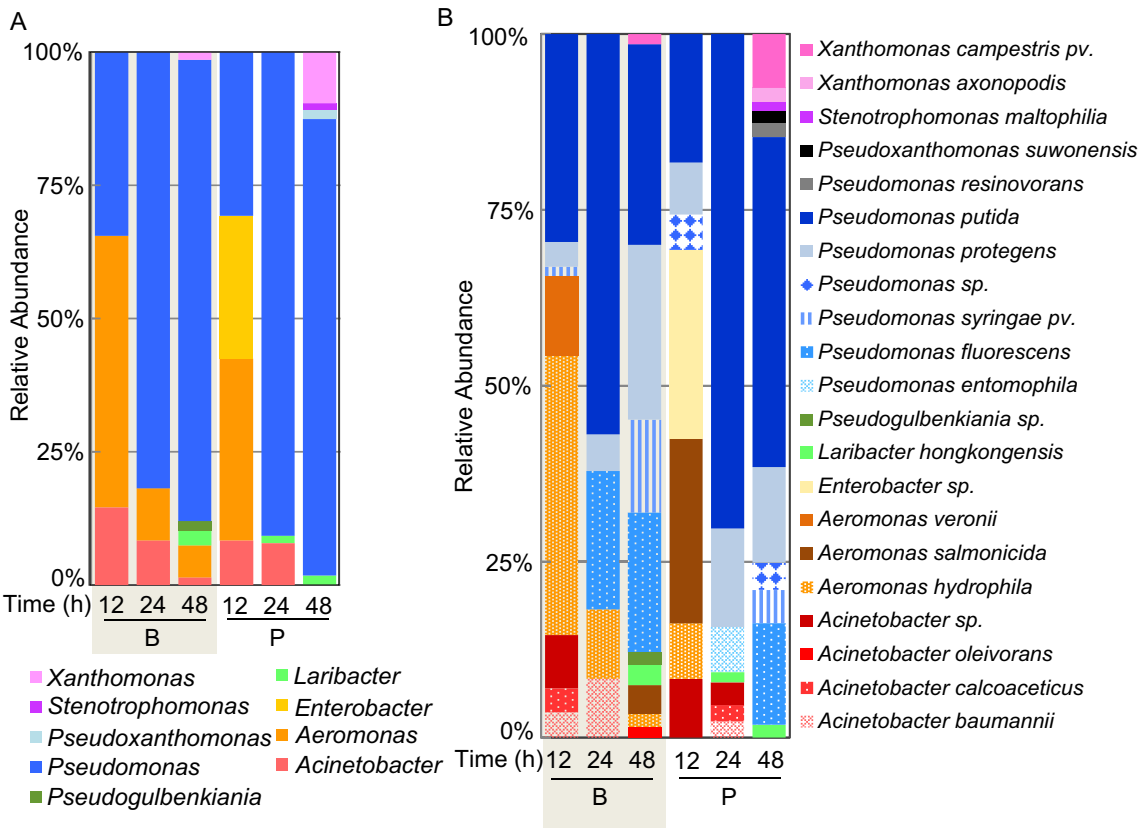

Fig. 3 Dynamic differences between planktonic and biofilm communities. **a** The relative abundance for each genus was calculated from the average of the number of reads assigned to *gyrB* (KEGG orthology K02470), *rpoB* (K03043), or *rplC* (K02906). "B" and "P" characters indicate biofilm and planktonic communities, respectively. **b** The relative abundance for each species was calculated in the same manner as for panel **a**

Table 1 Summary of upregulated genes in the biofilm

Biological categories and subcategories in KEGG database	Number of upregulated genes (in KEGG orthology)[a]	Number of genes detected in RNA-seq (in KEGG orthology)	Percentage of upregulated genes (%)[b]
Metabolism	273	1281	21.3
Carbohydrate metabolism	63	323	19.5
Amino acid metabolism	57	271	21.0
Energy metabolism	26	170	15.3
Lipid metabolism	26	82	31.7
Metabolism of co-factors and vitamins	23	122	18.9
Xenobiotics biodegradation and metabolism	23	60	38.3
Biosynthesis of other secondary metabolites	14	28	50.0
Nucleotide metabolism	14	105	13.3
Metabolism of other amino acids	13	56	23.2
Glycan biosynthesis and metabolism	8	31	25.8
Metabolism of terpenoids and polyketides	6	33	18.2
Environmental information processing	69	241	28.6
Membrane transport	46	140	32.9
Signal transduction	23	101	22.8
Genetic information processing	18	185	9.7
Replication and repair	12	62	19.4
Translation	4	84	4.8
Folding, sorting, and degradation	2	39	5.1
Human diseases	15	60	25.0
Infectious diseases: bacterial	6	32	18.8
Neurodegenerative diseases	4	15	26.7
Substance dependence	3	3	100
Cancers: overview	1	7	14.3
Cancers: specific types	1	3	33.3
Cellular processes	10	57	17.5
Cell motility	6	50	12.0
Transport and catabolism	4	7	57.1
Organismal systems	3	16	18.8
Nervous system	2	4	50.0
Endocrine system	1	12	8.3

[a] Upregulation in the biofilm is calculated as an average of fold-changes at three time points, and the number of genes in the KEGG orthology showing an upregulation of over threefold are included

[b] Calculated as the number of upregulated genes/number of genes detected in RNA-seq

biosynthesis (Fig. 4, see below for details) and iron-sulfur metabolism (*iscR*, *ycdN*, *iscA*, *hscB*, *ftnA*, *sitA*, *sitB*) were detected to a high extent. Upregulation of a number of stress response genes (*bolA*, *pspC*, *uspE*, *pspA*, *pspB*, *osmY*, *cpxP*, *osmE*, *treA*/*treF*) is reasonable, because a number of previous studies have reported that environmental stresses induce biofilm formation (O'Toole and Stewart 2005). In relation to the stress response, a functional cell envelope and peptidoglycan biosynthesis is necessary for cell attachment and biofilm maturation (Dong et al. 2011; Loo et al. 2000); therefore, induction of *pgm*, *amiA*, *yhcB*, *prc*, *rfbC*, *murC*, and *dacB* expression is assumed to be plausible according to the literature (see Tables 2, 3, 4, and 5). The expression of multiple genes for rRNA methyltransferases (*rlmB*, *rsmH*), chromosome partitioning (KEGG K03497, *mukB*), or leucine biosynthesis (*leuD*, *leuC*, *lrp*) was found to be upregulated, but the reason for this upregulation is unclear.

The downregulated genes in the biofilm community at all the time points are listed in Table 6, and the downregulated genes at one or two time point(s) are listed in Tables S4–S9. Downregulation of genes involved in the respiratory chain (*cyoB*, *cyoC*, *nuoI*, *cyoA*, *nuoM*, *cydB*/*appB*), nucleotide biosynthesis (*pyrD*, *purB*, *purN*, *preA*, *uraA*, *purK*, *purT*, *pyrF*), fatty acid metabolism (*fabF*, *fadR*, *fabD*, *fabH*, *accA*, *accD*), and DNA repair (*mutS*, *recF*, KEGG K03630, *dnaE*, *sulE*) was characteristic. Concerning the respiratory chain genes, this result may reflect that the interior of the biofilm is anaerobic. Anaerobic conditions in the biofilm are also suggested by the upregulation of *arcB* (Table 4), since ArcB represses the genes required for respiratory metabolism and the

Table 2 Upregulated genes in the biofilm community compared to the planktonic community at all the time points

KEGG orthology	Protein name	*E. coli* homologue	Fold change[a]			References related to biofilm
			12 h	24 h	48 h	
K00381	Sulfite reductase (NADPH) hemoprotein beta-component	CysI	5.9	6.1	2.7	Ren et al. 2005; Singh et al. 2015
K01738	Cysteine synthase A	CysK	5.8	3.8	2.8	Ren et al. 2005; Singh et al. 2015
K02417	Flagellar motor switch protein FliN/FliY	FliN	5.6	3.9	4.5	–
K02048	Sulfate transport system substrate-binding protein	CysP	5.4	5.8	8.6	Ren et al. 2005; Singh et al. 2015
K06134	Ubiquinone biosynthesis monooxygenase Coq7	none	4.9	5.1	3.9	–
K03218	23S rRNA (guanosine2251–2′-*O*)-methyltransferase	RlmB	4.8	4.0	4.1	–
K09908	Hypothetical protein (DUF1043 family inner membrane-anchored protein)	YhcB	4.8	3.8	3.6	Niba et al. 2007; Li et al. 2012
K01704	3-Isopropylmalate/(*R*)-2-methylmalate dehydratase small subunit	LeuD	4.0	3.7	4.2	–
K07126	None (Sel1 family TPR-like repeat protein)	YbeQ, YbeT, YjcO	3.8	4.6	4.2	–
K09773	Hypothetical protein (bifunctional regulatory protein: PEP synthase kinase and PEP synthase pyrophosphorylase)	PpsR	3.8	4.2	3.7	–
K01875	Seryl-tRNA synthetase	SerS	3.3	4.0	4.7	–
K01703	3-Isopropylmalate/(*R*)-2-methylmalate dehydratase large subunit	LeuC	3.3	5.8	4.6	–

[a] Log_2 of the number of reads in the biofilm samples divided by the number of reads in the planktonic samples. KOs whose fold changes exceed 2.0 at all the time points are listed

tricarboxylic acid cycle enzymes. Downregulation of the fatty acid metabolism genes listed above is consistent with upregulation of *fadE* expression (Table 3) because FadR is a positive and negative transcriptional regulator for fatty acid biosynthesis (*fab*) and fatty acid degradation (*fad*) genes, respectively in *E. coli* (My et al. 2013). We can conclude that fatty acid synthesis decreases during biofilm formation. Repression of DNA repair genes is expected to facilitate replication errors and increase the frequency of spontaneous mutations; since a biofilm acts as the evacuation spot from unfavorable environments, it may be involved in generating new genetic characters and diversity (see "Discussion" section for details). In addition, multiple genes for aminoacyl-tRNA synthase (*glnS*, *glyS*, *proS*, *valS*; note that *serS* is exceptionally upregulated) and thiamine biosynthesis (*thiG*, *thiI*) were downregulated, but the reason for this downregulation is unclear.

In concluding this section, many upregulated and downregulated genes were identified, and therefore, planktonic and biofilm communities were revealed to be in different dynamic states. The expression of many genes seemed to be consistent with their description in previous studies (references are indicated in Tables 2–6 and S1–S9), whereas some genes were newly identified as biofilm-inducible or biofilm-repressible in this study.

Cysteine and methionine biosynthesis

In Tables 2, 3, 4, 5, and 6 and S1–S9, a number of genes that are involved in cysteine and methionine biosynthesis are listed. The cysteine and methionine biosynthesis pathway is depicted in Fig. 4, and the regulation of individual genes upon biofilm formation is also shown. This figure shows that cysteine and methionine biosynthesis is upregulated and downregulated, respectively. Although the reason for this result is unclear, similar positive and negative effects of these amino acids on biofilm formation have been described earlier (Gnanadhas et al. 2015; Singh et al. 2015; Xu et al. 2014).

Table 3 Upregulated genes in the biofilm community compared to the planktonic community at 12 and 24 h

KEGG orthology	Protein name	*E. coli* homologue	Fold change[a]			References related to biofilm
			12 h	24 h	48 h	
K01256	Aminopeptidase N	PepN	6.3	4.4	−0.4	Allegrucci et al. 2006
K12256	Putrescine aminotransferase	None	5.9	5.5	1.4	Ding et al. 2014; Patel et al. 2006
K13643	Rrf2 family transcriptional regulator, iron-sulfur cluster assembly transcription factor	IscR	5.3	3.2	0.9	Roche et al. 2013; Wu and Outten 2009
K01835	Phosphoglucomutase	Pgm	5.2	3.6	1.3	Felek et al. 2010; Lazarevic et al. 2005
K14682	Amino-acid *N*-acetyltransferase	ArgA	5.1	4.4	0.0	Kolderman et al. 2015
K05527	BolA protein	BolA	4.8	4.2	−0.2	Dressaire et al. 2015; Vieira et al. 2004
K10806	Acyl-CoA thioesterase YciA	YciA	4.8	4.1	−0.1	–
K07243	High-affinity iron transporter	YcdN	4.3	3.5	0.0	–
K06445	Acyl-CoA dehydrogenase	FadE	4.2	3.9	1.8	Ojha and Hatfull 2007
K01448	*N*-Acetylmuramoyl-L-alanine amidase	AmiA	3.9	3.8	−0.5	Domenech et al. 2011; Heilmann et al. 1997
K03690	Hypothetical protein (conserved protein involved in ubiquinone-8 biosynthesis)	YigP	3.7	4.0	0.0	–
K02033	Peptide/nickel transport system permease protein	DdpB	3.6	2.9	0.0	–
K13628	Iron-sulfur cluster assembly protein	IscA	2.6	3.9	0.0	Roche et al. 2013; Wu and Outten 2009
K03797	Carboxyl-terminal processing protease	Prc	2.5	3.9	1.8	Dong et al. 2011

[a] Log_2 of the number of reads in the biofilm samples divided by the number of reads in the planktonic samples. KOs whose fold changes exceed 2.5 at 12 and 24 h and are between −0.5 and 2.5 at 48 h are listed

Regulation of flagellar genes

Previous studies have shown that the flagella play important roles in biofilm formation in both gram-negative and gram-positive bacteria (Belas 2014). The heat map presented in Fig. 5 shows the expression pattern of flagellar genes. In general, at the early stage of biofilm formation, many genes were upregulated, whereas in the middle stage, the appearance of downregulated genes was remarkable. At the late stage, the expression of genes that were downregulated at the middle stage seemed to be restored. This result is consistent with those of previous studies (Monds and O'Toole 2009; Okuda et al. 2012; Watnick and Kolter 2000). It is generally considered that at the early stage, flagella are necessary to sense and attach to solid surfaces and during biofilm growth, the flagella become obstructive as they destabilize biofilm structure. After biofilm maturation, it is assumed that flagella formation and flagella-associated motility again gains importance for escaping from the biofilm structures and to find new solid surfaces.

Discussion

From the metatranscriptome data (Tables 5, 3, 4, 5, and 6 and S1–S9), we speculated that the iron-dependent signaling mechanism triggers cells to form a biofilm and, as a consequence, genes related to stress response, cell envelope, flagella, and many others are regulated. Iron is reported to be important for *Pseudomonas* bacteria, both as a positive signaling molecule for biofilm formation and as a structural stabilizer for the biofilm matrix (Balaban 2008). In *E. coli*, iron-sulfur homeostasis and Fe-S cluster assembly are signals for biofilm formation (Roche et al. 2013; Wu and Outten 2009). These facts raise the question of why iron and sulfur are the chosen

Table 4 Upregulated genes in the biofilm community compared to the planktonic community at 12 and 48 h

KEGG orthology	Protein name	*E. coli* homologue	Fold change[a]			References related to biofilm
			12 h	24 h	48 h	
K00380	Sulfite reductase (NADPH) flavoprotein alpha-component	CysJ	6.4	0.0	5.7	Ren et al. 2005; Singh et al. 2015
K16961	Putative amino-acid transport system substrate-binding protein	None	6.2	0.0	5.2	–
K04774	Serine protease SohB	SohB	5.5	0.3	4.8	–
K07397	Putative redox protein	YhfA	5.4	0.0	4.4	–
K03497	Chromosome partitioning protein, ParB family	None	5.1	0.0	4.0	–
K04082	Molecular chaperone HscB (co-chaperone for [Fe-S] cluster biosynthesis)	HscB	5.0	0.0	3.7	Niba et al. 2007; Roche et al. 2013;
K03973	Phage shock protein C	PspC	5.0	0.0	4.1	Beloin et al. 2004; Chao et al. 2015; Domenech et al. 2013
K02518	Translation initiation factor IF-1	InfA	5.0	0.0	6.2	–
K03604	LacI family transcriptional regulator, purine nucleotide synthesis repressor	PurR	4.8	0.0	4.2	Knobloch et al. 2003; You et al. 2014
K02803	PTS system, *N*-acetylglucosamine-specific IIB component	NagE	4.8	0.0	5.0	Barnhart et al. 2006
K02804	PTS system, *N*-acetylglucosamine-specific IIC component	NagE	4.8	0.0	5.0	Barnhart et al. 2006
K01790	dTDP-4-dehydrorhamnose 3,5-epimerase	RfbC	4.7	0.0	3.2	Amini et al. 2009
K06212	Formate transporter	FocA	4.6	0.0	4.4	Beloin et al. 2004; Tremblay et al. 2013
K00956	Sulfate adenylyltransferase subunit 1	CysN	4.6	0.0	6.4	Ren et al. 2005; Singh et al. 2015
K14055	Universal stress protein E	UspE	4.5	0.0	3.3	Dressaire et al. 2015; Nachin et al. 2005
K03719	Lrp/AsnC family transcriptional regulator, leucine-responsive regulatory protein	Lrp	4.5	0.0	4.0	McFarland et al. 2008
K15551	Taurine transport system substrate-binding protein	TauA	4.4	0.0	6.9	Domka et al. 2007; Pysz et al. 2004
K00540	None (putative oxidoreductase)	YbbO, YohF, YeiQ, YghA	4.4	0.0	6.4	Ren et al. 2004
K16962	Putative amino-acid transport system permease protein	None	4.3	0.0	3.9	–
K07157	None (Lon-like protease)	None	4.1	0.0	3.8	–
K14062	Outer membrane protein N	OmpN	4.1	0.0	4.1	–
K03634	Outer membrane lipoprotein carrier protein	LolA	4.0	0.0	4.4	–
K00507	Stearoyl-CoA desaturase (delta-9 desaturase)	None	4.0	0.0	2.7	–
K02228	Precorrin-6A synthase	None	3.9	0.0	5.2	–
K07008	Glutamine amidotransferase	YafJ	3.9	0.0	5.0	–

Table 4 (continued)

KEGG orthology	Protein name	*E. coli* homologue	Fold change[a]			References related to biofilm
			12 h	24 h	48 h	
K07058	Membrane protein	YhjD, YihY	3.8	0.0	6.3	–
K03632	Chromosome partition protein MukB	MukB	3.8	0.0	3.6	–
K03969	Phage shock protein A	PspA	3.8	0.0	7.0	Beloin et al. 2004; Ito et al. 2008
K03970	Phage shock protein B	PspB	3.7	0.0	4.8	Beloin et al. 2004; Ito et al. 2008
K07726	Putative transcriptional regulator	YiaG	3.7	−0.1	2.9	–
K03438	16S rRNA (cytosine1402-N4)-methyltransferase	RsmH	3.6	0.4	3.2	–
K02217	Ferritin	FtnA	3.6	0.0	2.9	Ito et al. 2008
K07259	D-Alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase (penicillin-binding protein 4)	DacB	3.5	0.0	4.7	–
K00262	Glutamate dehydrogenase (NADP+)	GdhA	3.5	0.0	4.8	Ito et al. 2008; Kuboniwa et al. 2006
K09899	Hypothetical protein (conserved inner membrane protein)	YfbV	3.4	0.0	3.2	–
K01924	UDP-*N*-acetylmuramate–alanine ligase	MurC	3.4	0.9	3.3	–
K07648	Two-component system, OmpR family, aerobic respiration control sensor histidine kinase ArcB	ArcB	3.3	0.0	3.3	Longo et al. 2009; Post et al. 2014
K01147	Exoribonuclease II	Rnb	3.3	0.0	4.4	Pobre and Arraiano 2015
K10109	Maltose/maltodextrin transport system permease protein	MalF	3.2	0.0	3.2	–
K07071	None (putative NAD-dependent nucleotide-sugar epimerase)	YfcH	3.0	0.1	4.2	–
K06995	None (putative RmlC-like cupin)	None	2.9	0.0	3.3	–
K01082	3′(2′), 5′-Bisphosphate nucleotidase	CysQ	2.8	0.0	5.0	–
K12410	NAD-dependent deacetylase	CobB	2.6	0.0	4.9	Ito et al. 2008
K17103	CDP-diacylglycerol—serine *O*-phosphatidyltransferase	PssA	2.5	0.0	3.9	Chen et al. 2014; Russo et al. 2006

[a] Log_2 of the number of reads in the biofilm samples divided by the number of reads in the planktonic samples. KOs whose fold changes exceed 2.5 at 12 and 48 h and are between −0.5 and 2.5 at 24 h are listed

signaling molecules for biofilms. In primordial Earth, iron and sulfide were abundant, and the Fe-S cluster is thought to be one of the oldest and most versatile inorganic co-factors (Ayala-Castro et al. 2008; Lill 2009). In addition, Stoody and co-workers proposed the possibility that biofilms were the main form in which microbial cells existed in primordial Earth and that planktonic cells appeared much later during the evolutionary process (Stoodley et al. 2002). Taken together, it is natural that primitive microbes incorporated iron and sulfur in many of their cellular functions including biofilm formation and still utilize them as signaling molecules for transition between the biofilm and planktonic states.

Antimicrobials are widely used globally, but they give bacteria the opportunity to build resistance, allowing the emergence of resistant bacteria. Moreover, cells within biofilms are frequently tolerant to ordinary antimicrobials (Carvalhais et al. 2015; Stoodley et al. 2002). Therefore, the development of anti-biofilm drugs that do not inhibit cell growth is desirable. For some bacteria, iron chelators such as deferoxamine, lactoferrin, and 2,2′-bipyridyl are known to function as anti-biofilm drugs (Banin et al. 2005; Moreau-Marquis et al. 2009; Weinberg 2004). However, iron has a varied effect on bacterial biofilm formation; low iron concentration has a negative effect in *Pseudomonas*, *E. coli*, and *Vibrio cholerae* (Balaban

Table 5 Upregulated genes in the biofilm community compared to the planktonic community at 24 and 48 h

KEGG orthology	Protein name	*E. coli* homologue	Fold change[a]			References related to biofilm
			12 h	24 h	48 h	
K11604	Manganese/iron transport system substrate-binding protein	SitA	0.0	5.4	3.6	–
K00111	Glycerol-3-phosphate dehydrogenase	GlpA, GlpD	1.0	5.3	4.6	Tremblay et al. 2013
K04065	Hyperosmotically inducible periplasmic protein	OsmY	0.0	5.0	2.7	Giaouris et al. 2013; Waite et al. 2006; Zhang et al. 2007
K07122	None (phospholipid transport system transporter-binding protein)	MlaB	0.0	4.7	4.7	Ito et al. 2008
K06006	Periplasmic protein CpxP	CpxP	0.0	4.0	5.2	Beloin et al. 2004; Ma and Wood 2009
K04064	Osmotically inducible lipoprotein OsmE	OsmE	0.0	3.9	3.9	Waite et al. 2006
K11903	Type VI secretion system secreted protein Hcp	Hcp	0.0	3.9	4.7	Sha et al. 2013; Zhang et al. 2011
K01194	Alpha,alpha-trehalase	TreA, TreF	0.0	3.7	4.4	Hamilton et al. 2009
K07119	None (NADPH-dependent curcumin/ dihydrocurcumin reductase)	CurA	0.0	3.7	4.3	–
K11607	Manganese/iron transport system ATP-binding protein	SitB	0.0	3.6	3.7	–
K06955	None (uncharacterized protein)	none	0.0	3.6	6.3	–
K10555	AI-2 transport system substrate-binding protein	LsrB	0.0	3.3	3.4	Beloin et al. 2004; Novak et al. 2010; Shao et al. 2007

[a] Log_2 of the number of reads in the biofilm samples divided by the number of reads in the planktonic samples. KOs whose fold changes exceed 2.5 at 12 and 48 h and are between −0.5 and 2.5 at 24 h are listed

2008; Wu and Outten 2009), whereas a positive effect of iron is observed in *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Streptococcus mutants* (Balaban 2008). These observations suggest that, when removing undesired biofilms through manipulation of iron concentration, investigating the effect of iron on each biofilm is important. In this context, the metatranscriptome analysis approach described here would be effective for determining the effect of iron. The metatranscriptome data obtained in this study implied that iron had a positive effect on our biofilm model. Our preliminary experiments have shown that addition of 2,2′-bipyridyl to the starting culture indeed reduced the amount of biofilm without reducing bacterial growth (data not shown).

The targets of anti-biofilm drugs are not limited to iron and sulfur metabolism, and all genes regulated in the biofilms can be potential targets. For example, the expression of *treA*/*treF* was upregulated in our biofilm (Table 5), and the enzymatic activity of its gene product, α,α-trehalase, is known to be inhibited by casuarine or deoxynojirimycin (Cardona et al. 2009; Forcella et al. 2010). Similar candidates for anti-biofilm drugs are listed in Table S10, and we are now trying to develop novel tailor-made and target-oriented anti-biofilm drugs.

Downregulation of multiple DNA repair genes in the biofilm was observed in this study. Consistent with our result, it has been reported that *P. aeruginosa* cells within the biofilm community show up to 100-fold increased mutation frequency compared to that in corresponding planktonic cells (Driffield et al. 2008; Luján et al. 2011; Oliver et al. 2000). In addition, bacteria possess a mechanism of stress-induced mutagenesis in which error-prone DNA polymerases are upregulated and error-correcting enzymes are downregulated under stress conditions (Al Mamun et al. 2012; Foster 2007). These facts suggest that biofilm formation is a means for adaptive evolution and generating phenotypic diversity. This idea is further supported by the fact that many biofilm structures contain extracellular DNA, which is a source of genes in horizontal gene transfer (Dominiak et al. 2011; Madsen et al. 2012) and that *Streptococcus mutans* cells grown in biofilms have a 10-

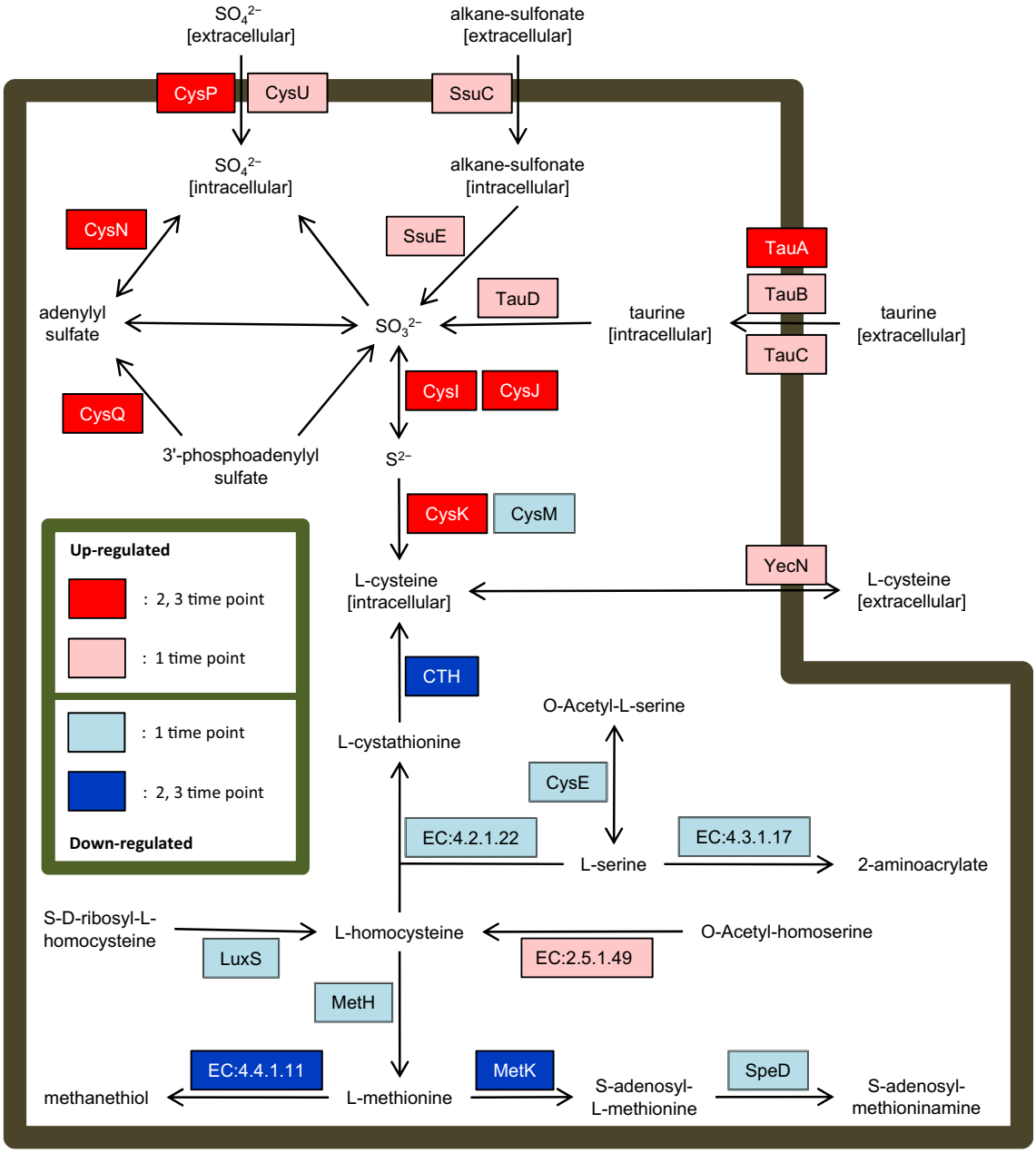

Fig. 4 Regulation of cysteine and methionine biosynthesis. The cysteine and methionine biosynthesis pathway is shown. *Dark* and *light red colors* indicate genes whose expression is upregulated at least at two time points and one time point, respectively. *Dark* and *light blue colors* indicate the downregulated genes at least at two time points and one time point, respectively

to 600-fold higher rate of natural transformation than planktonic cells (Li et al. 2001).

In addition to cysteine, methionine, and leucine biosynthesis genes (see the "Results" section), glutamate (*gdhA*, Table 4, upregulated), histidine (*hisH*, Table 6, downregulated), proline (*proB*, Table S5, downregulated), and arginine (*argE*, Table S6, downregulated) biosynthesis genes were identified as biofilm-regulated genes. The positive effects of cysteine and leucine and the negative effects of methionine and arginine on biofilm formation are in good agreement with observations from previous studies (Kolderman et al. 2015; McFarland et al. 2008; Noothalapati Venkata et al. 2011; Singh et al. 2015). The mechanism involved in the effect of amino acids on biofilm formation is unclear, but they are attractive anti-biofilm drugs because of their safety for human health. Intriguingly, high levels of leucine were found to accumulate within microcolonies in *E. coli* biofilms (Noothalapati Venkata et al. 2011), implying that leucine is a functional component of biofilm structures or a signaling molecule for biofilm formation. Upregulation of *lrp* (encoding leucine-responsive regulatory

Table 6 Downregulated genes in the biofilm community compared to the planktonic community at all the time points

KEGG orthology	Protein name	*E. coli* homologue	Fold change[a]			References related to biofilm
			12 h	24 h	48 h	
K07665	Two-component system, OmpR family, copper resistance phosphate regulon response regulator CusR	CusR	−5.5	−3.5	−3.8	–
K02501	Glutamine amidotransferase	HisH	−4.7	−3.2	−4.7	Ito et al. 2008
K09136	Ribosomal protein S12 methylthiotransferase	YcaO	−4.4	−4.6	−4.6	Tenorio et al. 2003
K02523	Octaprenyl-diphosphate synthase	IspB	−4.2	−4.6	−4.8	–
K00254	Dihydroorotate dehydrogenase	PyrD	−4.1	−5.1	−4.9	Ito et al. 2008; Pettigrew et al. 2014; Ueda et al. 2009
K02616	Phenylacetic acid degradation operon negative regulatory protein	PaaX	−4.1	−4.0	−4.0	–
K01758	Cystathionine gamma-lyase	None	−4.1	−4.0	−4.0	Frese et al. 2013
K01756	Adenylosuccinate lyase	PurB	−4.1	−3.6	−2.6	Chauhan and Nautiyal 2010, Ge et al. 2008;
K09796	Hypothetical protein	none	−4.1	−4.8	−4.9	–
K03149	Thiamine biosynthesis ThiG	ThiG	−4.1	−4.5	−5.0	Pysz et al. 2004; Yu et al. 2015
K03555	DNA mismatch repair protein MutS	MutS	−3.9	−3.5	−4.5	Driffield et al. 2008; Luján et al. 2011
K13788	Phosphate acetyltransferase	Pta	−3.9	−5.6	−3.1	Kim et al. 2015; Wolfe et al. 2003
K03473	Erythronate-4-phosphate dehydrogenase	PdxB	−3.9	−4.1	−4.5	–
K00931	Glutamate 5-kinase	ProB	−3.9	−2.0	−4.8	–
K01886	Glutaminyl-tRNA synthetase	GlnS	−3.8	−4.0	−5.1	–
K11891	Type VI secretion system protein ImpL	none	−3.8	−4.4	−3.8	–
K09458	3-Oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]	FabF	−3.8	−3.5	−2.1	–
K03110	Fused signal recognition particle receptor	FtsY	−3.6	−4.6	−4.0	Shemesh et al. 2010
K03595	GTP-binding protein Era	Era	−3.5	−5.1	−4.2	–
K01070	*S*-Formylglutathione hydrolase	YeiG	−3.3	−5.8	−4.8	–
K02298	Cytochrome o ubiquinol oxidase subunit I	CyoB	−3.0	−3.1	−6.8	Ito et al. 2008
K02299	Cytochrome o ubiquinol oxidase subunit III [EC:1.10.3.-]	CyoC	−2.3	−5.9	−3.6	Ito et al. 2008
K03629	DNA replication and repair protein RecF	RecF	−2.0	−3.5	−6.1	–

[a] Log_2 of the number of reads in the biofilm samples divided by the number of reads in the planktonic samples. KOs whose

protein, Table 4) supports the signaling molecule hypothesis, because Lrp activates transcription from many fimbrial genes that depend on leucine (Noothalapati Venkata et al. 2011).

EPS is a key component for maintaining biofilm architecture, possibly acting as an intercellular cement. Quorum sensing positively regulates EPS production and facilitates the development of mixed species populations (Madsen et al. 2012). LsrB (Table 5, upregulated) is a subunit of the transporter complex for the autoinducer-2 molecule, which is produced by many bacterial species and is one of the quorum-sensing signaling

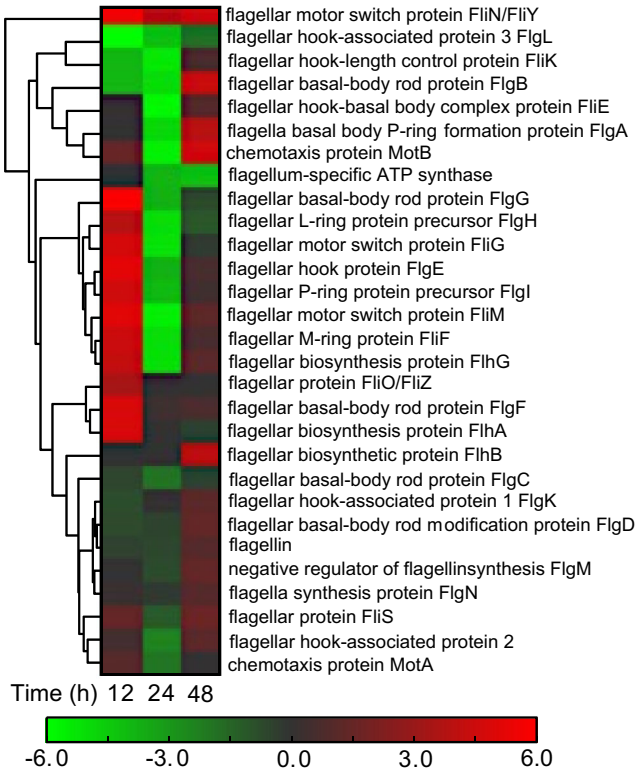

Fig. 5 Heat map of expression pattern for flagella-related genes. The 29 genes indicated were detected out of 37 genes in the KEGG pathway KO02040 (Flagellar assembly) from the RNA-seq data. The heat map legend at the bottom indicates the color values corresponding to transcription levels, with upregulation shown in *red* and downregulation in *green*

molecules (Shao et al. 2007). In addition, PssA (Table 4, upregulated) catalyzes the first step of EPS synthesis (Chen et al. 2014), and a *pssA* mutation in *Rhizobium leguminosarum* results in complete inability to form biofilms (Russo et al. 2006). Collectively, we conclude that EPS plays an important role in our multi-species biofilm community. However, the EPS produced by some bacteria is also known to interfere with biofilm formation by other bacteria, and some bacteria produce enzymes to degrade quorum-sensing molecules possibly to interfere with the inclusion of unfavorable bacteria in the biofilm (Madsen et al. 2012). Since the generic compositions of planktonic and biofilm communities were almost indistinguishable in this study (Fig. 1b), it seemed that there were no such interference mechanisms in this case.

Phosphotransacetylase (Pta, Table 6, downregulated at all the time points) and acetate kinase (AckA, Table S3, upregulated only at 48 h) are enzymes that convert acetyl-CoA to acetyl-phosphate and acetyl-phosphate to acetate, respectively. The role of these proteins on biofilm formation has been discussed in many bacteria, but both positive and negative effects have been reported. A consensus view is that acetyl-phosphate functions as a signaling molecule through two-component response regulators (possibly, as a phosphate donor) and its intracellular level influences biofilm formation (Kim et al. 2015; Wolfe et al. 2003). In *E. coli*, the acetyl phosphate level affects the expression of about 100 genes including those related to flagella synthesis, type 1 pili, capsule, and stress effectors (Wolfe et al. 2003). In our biofilm community, the intracellular acetyl-phosphate level is expected to be lower than that in the planktonic community. Many genes for two-component response regulators are listed in Tables 2, 3, 4, and 5 and in S1–S9, and thus, the expression of these genes may be regulated by acetyl-phosphate. As discussed above, iron, leucine, and autoinducer-2 may also act as signaling molecules for biofilm formation. Studies on the interplay between these signaling molecules are the next important issue.

For upregulated and downregulated genes, it is necessary to determine expression dynamics accurately using quantitative real-time RT-PCR. However, quantification of these genes according to KO is difficult, because multiple orthologous genes are assigned to one KO and a universal primer set to amplify all the orthologous genes is not designed. One solution is quantifying each orthologous gene separately and combining the results. In addition, quantifying the amount of proteins expressed from the upregulated and downregulated genes is important in the future.

The multi-species biofilm has been poorly studied so far, because it is too complex to understand overall activities and functions. However, recent advances in next-generation sequencer have enabled us to study the multi-species biofilm as a system without investigating individual microbial cells. There is, however, still one hurdle; examples of multi-species biofilm in nature, marine sediments, chronic wounds, and dental plaques are well known (Si et al. 2015; Yang et al. 2011), but none of these biofilms can be maintained easily, safely, and over a long duration. Therefore, we believe that a study on reproducing multi-species biofilms in the laboratory is important to quantitatively and repeatedly compare planktonic and biofilm samples. The biofilm model used in this study may differ from the original biofilm because of its culture in rich media (Fig. 1b). It is important to establish model biofilms wherein microbial populations are maintained in a nature-identical manner for deeper understanding of biofilms and for designing tailor-made anti-biofilm drugs.

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Compliance with ethical standards This article does not contain any studies with human participants or animals.

Conflict of interest The authors declare that they have no conflicts of interest.

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