Identification of specific protein amino acid substitutions of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* ST131: a proteomics approach using mass spectrometry

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The global pandemic of ESBL-producing *Escherichia coli* is associated with sequence type 131 (ST131). However, mechanisms of ST131 spread remain unclear. This study searched for proteins with amino acid substitutions specific for ST131 and used proteomics analysis to clarify ST131 characteristics. Five proteins had ST131-specific amino acid substitutions: uncharacterized protein YahO with E34A (m/z 7655); UPF0337 protein YjbJ with V59D, D60S and T63K (m/z 8351); uncharacterized protein YnfD with S106T (m/z 8448); and acid stress chaperone HdeA with Q92K and N94S (m/z 9714). Soluble cytochrome b562 (m/z 11783) showed seven amino acid substitutions, and the sequence differed between clade C of the pandemic clade and non-C. *In silico* analysis showed YahO protein-protein interaction with YjbJ, possibly related to biofilm formation. Although the function of soluble cytochrome b562 is electron transport of unknown function, its involvement in biofilm formation was predicted. HdeA was a gastric acid resistance-related protein. The function of YnfD was completely unclear. In conclusion, ST131-specific protein amino acid substitutions consisted mainly of a gastric acid resistance protein and proteins of unknown function (possibly involved in biofilm formation), which might be mechanisms for long-term colonization in the human intestinal tract.
current pandemic clones are strains classified into the C clade by whole genome analysis. Particularly, clades C1 and C2 are prevalent worldwide. In addition, the C1-M27 subclade with blaCTX-M-27 became especially prominent after 2009. In this way, the population structure of ST131 has been explored extensively by many researchers. However, the reasons for the success of ST131 as an antimicrobial-resistant pandemic clone have not been clarified. The purpose of this study was to search for the proteins with specific amino acid substitutions for these clones using MALDI-TOF MS and identify them to clarify the characteristics of ST131 and its mechanisms of spread.

Results
The search for specific proteins and their amino acid substitutions of ST131 was performed by MALDI-TOF MS analysis. Table 1 shows the results of the statistical analysis of the peaks of ST131 and non-ST131 using the Wilcoxon rank sum test of ClinProTools software. Ten peaks of m/z 3236, 4176, 4857, 7655, 8351, 9710 and 11783 were detected as reproducible specific peaks of ST131. The peaks of m/z 3236, 4176, 4857, 5381, 6827, 7655, 8351, and 8448 were detected 3 out of 3 times, whereas those of m/z 9710 of HdeA, and the m/z 8351 of YjbJ. The m/z 8448 peak was identified as uncharacterized protein YnfD (Uniprot accession no. P0AES9) belonging to the HdeA family. The domain was HdeA, and the function is stress response protein acid-resistance protein. The m/z 11783 peak was identified as soluble cytochrome b562 (Uniprot accession no. P0ABE7) belonging to the cytochrome_b562 family. The mature chain domain was cytochrome_B562, and the function is also unknown. The m/z 9710 peak was identified as acid stress chaperone HdeA (Uniprot accession no. P0AES9) belonging to the HdeA family. The domain was HdeA, and the function is stress response protein acid-resistance protein. The m/z 11783 peak was identified as soluble cytochrome b562 (Uniprot accession no. P0ABE7) belonging to the cytochrome_b562 family. The mature chain domain was cytochrome_b562, and the function is electron-transport of unknown function. The m/z 3236 and 4857 peaks were multivalent ions of m/z 9710 of HdeA, and the m/z 4176 peak was a multivalent ion of m/z 8351 of YjbJ. The m/z 5381 and 6827 peaks were not identified in this study.

Identification of proteins with specific peaks in ST131 by LC-MS/MS. Table 2 shows the ST131 proteins with specific peaks identified using LC-MS/MS and their characteristics based on the Uniprot database. Five of the 10 specific peaks of ST131 were identified in this study. The m/z 7655 peak was identified as uncharacterized protein YahO (Uniprot accession no. P75694) belonging to the Bhs/McbA family. The chain domain of the YahO protein was DUF1471, and its function is unknown. The m/z 8351 peak was identified as UPF0337 protein YjbJ (Uniprot accession no. P68206) belonging to the UPF0337 (CsbD) family. The domain of YjbJ protein was CsbD, and its function is unknown. The m/z 8448 peak was identified as uncharacterized protein YnfD (Uniprot accession no. P0ABE7), and its family was unclear. The chain domain of YnfD protein was DUF1161, and its function is also unknown. The m/z 9710 peak was identified as acid stress chaperone HdeA (Uniprot accession no. P0AES9) belonging to the HdeA family. The domain was HdeA, and the function is stress response protein acid-resistance protein. The m/z 11783 peak was identified as soluble cytochrome b562 (Uniprot accession no. P0ABE7) belonging to the cytochrome_b562 family. The mature chain domain was cytochrome_b562, and the function is electron-transport of unknown function. The m/z 3236 and 4857 peaks were multivalent ions of m/z 9710 of HdeA, and the m/z 4176 peak was a multivalent ion of m/z 8351 of YjbJ. The m/z 5381 and 6827 peaks were not identified in this study.

Comparison of amino acid sequences of identified proteins between ST131 and non-ST131. Figure 1 shows the amino acid sequences and substitutions specific for ST131. The YahO-specific substitution of ST131 was E34A in the chain part outside the DUF1471 domain, and the rates were 98.0% (96/98) in ST131 including all clades and 14.1% (14/99) in non-ST131. The YjbJ-specific substitutions of ST131 were V59D, D60S and T63K in the chain part inside the CsbD domain, and the rates were 100% (98/98) in ST131 including all clades and 36.4% (36/99) in non-ST131. Moreover, we found seven amino acid substitutions specific for ST131 in the chain part inside the cytochrome_B562 domain of soluble cytochrome b562. In addition, the C1 and C2 subclades of the ST131 pandemic clade and the A and B clades of the ST131 non-pandemic clade differed in amino acid sequences of soluble cytochrome b562. The amino acid substitutions specific for the pandemic clade were T35N, V39I, VorT46A, V66G.
which is a biofilm formation regulator. Currently known function using the Uniprot database (http://www.uniprot.org). bUnknown due to protein detection. However, no protein identification was performed in these previous studies. We also detected origins and isolation dates, several peaks of ST131 similar to those in the present study including E. coli. In a study using 109 ESBL-producing m/z was identified as 9713. Moreover, in a study using 73 E. coli collected from clinical settings and the environment in France, the peak m/z 9710, which was similar to the peaks detected in these reports, and revealed it to be gastric acid resistance-related protein. hdeA was included in the cluster mainly composed of gastric acid resistance-related proteins. The ynfD was a node isolated from those clusters. The cybC had an interaction with tomB, which is a biofilm formation regulator.

**M54K, G72D, E76D and S122A**, which were possessed by all C clades. The amino acid substitutions most specific for ST131 were E34A of YahO followed by various substitutions of soluble cytochrome b562.

**In silico prediction of protein-protein interactions.** Figure 2 shows the identified proteins of ST131 predicted to have protein-protein interaction by the STRING database (organism database: *Escherichia coli* K12 MG1655). The hdeA was included in the cluster mainly composed of gastric acid resistance-related proteins such as hdeB, gadA, gadB and gadC. Besides, it was predicted that yahO and yjbJ might indicate protein-protein interaction, and their function was unknown, including that of its neighbouring proteins. The ynfD was a node isolated from those clusters. The cybC was not detected in this database. Figure 3 shows the results of a protein search with the domain structure common to YahO DUF1471 using GeneMANIA. The bsmA and bhsA have the DUF1471 domain as does YahO, and their function was related to biofilm formation. Furthermore, mcba was a node that had an interaction with YahO. The cybC had an interaction with tomB, which is a biofilm formation regulator.

**Discussion**

The purpose of this study was to search for and identify the proteins with amino acid substitutions specific for ST131 by proteomics analysis and to clarify the characteristics of ST131 to identify clear mechanisms for its spread. In this study, we found several proteins with the amino acid substitutions specific for ST131 and predicted their protein-protein interactions by bioinformatics using various web-based databases.

**MALDI-TOF MS typing of ST131** has been explored extensively by many researchers. In a study using 149 ESBL-producing *E. coli* collected from seven acute-care hospitals in the Kyoto and Shiga regions of Japan, the most specific peak of ST131 was m/z 9720, with a sensitivity of 97.0% and a specificity of 91.5%. Also, in a study using 109 ESBL-producing *E. coli* collected from clinical settings and the environment in France, the peak was identified as m/z 9713. Moreover, in a study using 73 *E. coli* collected from different settings, geographic origins and isolation dates, several peaks of ST131 similar to those in the present study including m/z 9713 were detected. However, no protein identification was performed in these previous studies. We also detected m/z 9710, which was similar to the peaks detected in these reports, and revealed it to be gastric acid resistance-related protein HdeA and found an amino acid substitution specific for ST131. HdeA localizes in the bacterial periplasm and exhibits acid resistance by expressing chaperone-like activity that suppresses denaturation of proteins under acidic conditions. Although it is unclear how this amino acid substitution affects the strains, ST131 might have high resistance to gastric acid that allows it to reach the human intestinal tract.

In this study, we found YahO protein showing a m/z 7650 peak that is more specific than HdeA. We already found in our earlier study that YahO is a protein specific for ST131. We predicted its function using the STRING and GeneMANIA databases, which predicted YahO to have protein-protein interaction with YjbJ, another protein we found, suggesting that they might be related to biofilm formation. Kudinha et al. and Clermont et al. both found that the prevalence of strains producing biofilms was greater among ST131 than among non-ST131 clinical strains. Besides, Eletsky et al. researched the function of the DUF1471 domains of *Salmonella* protein YahO, and they concluded that YahO is closely related to YcrF/BhsA, YblM/McbA, Yjfo/BlmA or YcrF, which are associated with a stress response and biofilm formation. This was also predicted in our study. In the future, it will

### Table 2. ST131-specific proteins identified using LC-MS/MS and their characteristics based on the Uniprot database.

| Specific peaks (m/z) | Protein name | Predicted protein main localization | Coding gene | Family and domains | Function |
|----------------------|--------------|-------------------------------------|-------------|-------------------|----------|
| 3236                 | Acid stress chaperone HdeA (trivalent ion of 9710 m/z) | — | — | — | — |
| 4176                 | UPF0337 protein YjbJ (divalent ion of 8351 m/z) | — | — | — | — |
| 4857                 | Acid stress chaperone HdeA (divalent ion of 9710 m/z) | — | — | — | — |
| 5381                 | Not identified | s/o cytosolb | — | — | — |
| 6827                 | Not identified | s/o cytosolb | — | — | — |
| 7655                 | Uncharacterized protein YahO | Periplasm | yahO | YahO family, DUF1471 | Unknown |
| 8351                 | UPF0337 protein YjbJ | Cytosol | yjbJ | UPF0337 (CsbD) family, CsbD | Unknown (predicted stress-induced protein) |
| 8448                 | Uncharacterized protein YnfD | s/o periplasm^b | ynfD | Unknown, DUF1161 | Unknown |
| 9710                 | Acid stress chaperone HdeA | Periplasm | hdeA | HdeA family, HdeA | Stress response protein acid-resistance protein |
| 11783                | Soluble cytochrome b562 | Periplasm | cybC | Cytochrome b562 family, cytochrome_b562 | Electron-transport protein of unknown function |

**Note:** The proteins were investigated for protein name, localization, coding gene, family and domain, and currently known function using the Uniprot database (http://www.uniprot.org). **b**Unknown due to protein unidentified or not registered in the Uniprot database. Estimated from the fraction from which the proteins were extracted in this study.
be necessary to elucidate how this specific amino acid substitution of YahO and YjbJ affects biofilm formation. In addition, in the cybC sequence of soluble cytochrome b562, we found a specific sequence in the pandemic clade C. Moreover, cybC had an interaction with tomB, which is a biofilm formation regulator. These proteins possibly involved in biofilm formation may be related to the affinity of ST131 for humans.

The characteristics of ST131 clarified in this study might allow a hypothesis of the mechanism of colonization in the human intestinal tract. We hypothesized that ST131 does not die when passing through the stomach because of its resistance to gastric acid, and when it reaches the intestinal tract, it survives for a long time by

Figure 1. The amino acid sequence and its substitutions specific for ST131. The amino acid sequences of 1) uncharacterized protein YahO, 2) UPF0337 protein YjbJ, 3) uncharacterized protein YnfD, 4) acid stress chaperone HdeA and 5) soluble cytochrome b562 are shown.
forming a biofilm in the intestinal tract. As this is only a hypothesis, it will be necessary to elucidate the influence of these amino acid substitutions in the future.

This study has several limitations. First, the ST131 and non-ST131 strains used in this study were collected only from various regions in Japan and not sites worldwide. However, the strains were collected from all over Japan and were not localized to specific regions. Because the strain characteristics, such as fimH30 typing and the MALDI data, are consistent with those of other previous studies, we believe the findings are reliable. Second, as stated previously, although we clarified the difference in amino acid substitutions in various proteins between ST131 and non-ST131, the influence of these substitutions remains unclear. In the future, we will perform function analysis, such as pull-down and genome editing assays, of these proteins. Finally, although YnfD was identified as a protein with an amino acid substitution specific for ST131, its function could not be deduced at all.

In conclusion, we found several proteins with amino acid substitutions specific for ST131. They consisted mainly of gastric acid resistance proteins and proteins with unknown function (but which were estimated to be

**Figure 2.** Protein-protein interaction of various proteins predicted using the STRING database (https://string-db.org). The organism database used *Escherichia coli* K12 MG1655. The STRING interaction map was generated using default settings (medium confidence of 0.400; criteria for linkages are neighbourhood, gene fusion, co-occurrence, co-expression, experiments, databases and text mining). The circles indicate the coding genes of proteins identified in this study.

**Figure 3.** The results of the protein search with a domain structure common to YahO DUF1471 using GeneMANIA (https://genemania.org). The *bsmA* and *bhsA* are the coding genes of biofilm related proteins, and *mcbA* is the coding gene of colonic acid mucoidy stimulation protein. The *yjfN*, *ybiI*, *yhcN*, *yjfY*, *ykgl* and *ydgH* are the coding genes of hypothetical proteins.
biofilm formation proteins). These proteins might be associated with the mechanism of long-term colonization of ST131 in the human intestinal tract. In the future, by clarifying the functions of these proteins and their substitutions, the mechanism behind the worldwide pandemic of the ST131 might be further clarified.

**Materials and Methods**

**Bacterial isolates.** To search for proteins with specific amino acid substitutions for ST131, 197 ESBL-producing *E. coli* (97 strains of ST131 and 100 strains of non-ST131) isolated from clinical specimens collected at 24 clinical facilities in Japan between 2011 to 2013 were used. The 24 clinical facilities comprised 23 acute-care hospitals and 1 commercial laboratory located throughout Japan: 18 facilities from Western Japan and 6 facilities from Eastern Japan. These samples, which were derived from our previous study\(^7\) (n = 74) and the present study (n = 123), were kindly provided by the Association of Japan Community Healthcare Organization (JCHO) hospitals and the Study of Bacterial Resistance in the Kinki Region of Japan (SBRK) and were randomly extracted (Table 3).

These strains were characterized by ST131 typing, ST131 clade typing, O25b serotyping, *fimH* subclonal typing and β-lactamase gene typing. Bacterial DNA was purified using a QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). In ST131 typing, ST131 was defined based on PCR detection of ST131-specific SNPs in the *mdh* and *gyrB* alleles\(^7\). In addition, the ST131 clade and subclade were defined based on multiplex PCR using seven specific SNP primers\(^7\). In O25b serotyping and *fimH*30 subclone typing, specific PCR detection based on each specific primer was performed\(^9,23\). In addition, *fimH*30-Rx was defined based on PCR detection of ybbW SNP typing\(^7\). In β-lactamase gene typing, strains were analysed to determine the presence of ESBL encoded by *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>CTX-M-1</sub>-like, *bla*<sub>CTX-M-8</sub>-like and *bla*<sub>CTX-M-9</sub>-like\(^{25,26}\). In addition, PCR direct sequencing analysis was performed on *bla*<sub>SHV</sub>- and *bla*<sub>TEM</sub>-positive strains\(^7\).

**MALDI-TOF MS data analysis.** The method of MALDI-TOF MS analysis followed that of our previous study\(^7\). The strains were cultured at 37°C for 16-24 h using 5% sheep blood agar. We performed ethanol-formic acid protein extraction from grown colonies for preparation of the MALDI-TOF MS analysis and used Bruker Bacterial Test Standard (Bruker Daltonik, Bremen, Germany) for calibration. MALDI-TOF MS analysis was performed using MALDI Biotyper (Bruker Daltonik). Spectra obtained by MALDI-TOF MS analysis were used for comparison of the
For further proliferation, the proteins whose functions were not predicted on STRING were predicted by investigating protein-protein interaction were identified by STRING database version 10.5 (https://string-db.org).

**Table 3.** Characteristics of the O25b, fimH30 and ESBL genotypes in ESBL-producing *E. coli* used in this study. *Numbers in parentheses indicate the number of samples.

| Sequence type | ST131 clade | ST131 subclade | O serotypes and fimH types | ESBL gene |
|---------------|-------------|---------------|-----------------------------|-----------|
| ST131 (97)    | C (85)      | C2 (34)       | O25b, fimH30Rx (34)         | CTX-M-1 group [M-15] (33), M-1 [M-15] + M-9 group (1) |
|               |             | C1-M27 (33)   | O25b, fimH30 non-Rx (30)    | CTX-M-9 group (29), M-1 group [M-15] (1) CTX-M-9 group (3) |
|               |             | C1-nM27 (18)  | O25b, fimH30 non-Rx (16)    | CTX-M-9 group (10), M-2 group (5), M-1 group [M-15] (1) CTX-M-9 group (2) |
| A (7)         | —           | —             | O16, fimH30 negative (5)    | CTX-M-9 group (4), M-1 group [non-M-15] (1) CTX-M-9 group (1) CTX-M-9 group (1) |
| B (5)         | —           | —             | O25b, fimH30 negative (5)   | CTX-M-2 group (2), M-1 group [non-M-15] (2), SHV-12 (1) |
| Non-ST131 (100)| —          | —             | —                           | CTX-M-9 group (45), M-1 group (39), M-2 group (11), SHV-12 (5), SHV-2 (1) |

**Proteomic analysis.** The identification of the proteins with mass peaks specific to ST131 followed that of our previous study. The strains with these specific peaks were incubated using 10 L LB broth, and then all pellets were collected after centrifugal separation. Next, to collect periplasmic proteins, the pellets were suspended with 30 mM Tris–HCl pH 8.0 with 20% sucrose on ice and incubated for 10 min at room temperature with slow mixing. Then, after centrifugal separation, the pellets underwent osmotic shock for 10 min using 5 mM MgSO4 solution on ice. After centrifugal separation, the supernatant was used as the periplasmic proteins fraction after being dialyzed against 20 mM Tris–HCl pH 8.0. The pellets were suspended with 20 mM Tris–HCl pH 8.0, and soluble cytoplasmic proteins were collected after sonication of the pellets for 15 min. After centrifugal separation, the supernatant was used as the soluble cytoplasmic proteins fraction after being dialyzed against 20 mM Tris–HCl pH 8.0. Then, each of the protein fractions was purified by ion-exchange chromatography using Macro-Prep diethylaminoethyl support (Bio-Rad Laboratories, Inc., Hercules, CA) and reversed-phase HPLC using TSKgel ODS-100V (Tosoh Corporation, Tokyo, Japan). Finally, the gel obtained by tricine–SDS-PAGE with silver staining was analysed by LC-MS/MS using a High-Performance Liquid Chromatograph Ion-trap Time-of-Flight mass spectrometer (Shimazu Corporation, Kyoto, Japan) after in-gel digestion with trypsin of the target protein band was conducted. Trypsin digestion was performed using 10 µg/mL of sequencing grade modified trypsin (Promega KK, Tokyo, Japan), and the trypsin solution was incubated at 37 °C for 15h. Spectra obtained from bottom-up proteomics were analysed using Mascot MS/MS Ion Search (Matrix Science K.K., Tokyo, Japan) to search and assign the obtained peptides to the SwissProt database. The identified proteins were investigated for protein name, localization, coding gene, family and domain, and currently known function using the Uniprot database (http://www.uniprot.org).

**Genomic analysis.** From the amino acid sequencing identified by LC-MS/MS, the forward and reverse primers of each target protein were designed (Table S1), and comparison of amino acid sequences translated from nucleotide sequences between ST131 and non-ST131 was performed by PCR direct sequencing. Seaview software version 4 (http://phylob.univ-lyon1.fr/software/seaview) was used for sequence alignment and comparison. Also, the theoretical molecular weight calculated from the amino acid sequences was computed using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/).

**In silico prediction of protein-protein interactions.** The ST131 proteins predicted to have protein–protein interaction were identified by STRING database version 10.5 (https://string-db.org). Furthermore, the proteins whose functions were not predicted on STRING were predicted by investigating protein domain common proteins using GeneMANIA (https://genemania.org).

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Author Contributions

Conceived and designed the experiments: A.N. and M.K. Performed the experiments: A.N., Y.O. and N.N. Analysed the LC-MS/MS: N.H. Interpreted the proteomics data: A.K. and N.H. Drafted the manuscript: A.N. and M.K. Provided critical input and approved the final manuscript: All authors.

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