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Method Article

Liquid chromatography mass spectrometry-based proteomics of *Escherichia coli* single colony

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A B S T R A C T

The *Escherichia coli* proteome is the most extensively characterized and studied of all prokaryotic proteomes. Despite this, large scale bacterial proteomics experiments performed on *E. coli* cells grown in liquid cultures have failed to identify key virulence factors thought to be important determinants in establishing bacterial infections. It seems likely that many important determinants associated with virulence and host cell adhesion are exclusively expressed during growth in biofilms, which can be crudely mimicked on solid media. This method describes a simple workflow to characterize the unique proteome signature of individual, isolated single colonies, using *E. coli* K12 strain grown on solid media as a model system. The workflow thus provides a means to explore the proteomes of minimally passaged clinical isolates of bacteria grown on primary culture plates and to identify both unique and differentially expressed proteins contained therein.

Value of the method:

- Simple mass spectrometry-based proteomics workflow to characterise the proteome of single colony forming units
- Enables exploration of the proteomes of minimally passaged clinical isolates from primary culture plates
- Identification of virulence factors expressed in true or mimicked biofilms that may be missed in liquid cultures

Method name: *E. coli* single colony proteome analysis

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Subject area	Microbiology
More specific subject area	Bacterial proteomics
Method name	*E. coli* single colony proteome analysis
Name and reference of original method	This single colony proteomics analysis was applied to a biological question in our recent study "Comparison between the proteome of Escherichia coli single colony and during liquid culture": doi: 10.1016/j.jprot.2020.103929 Partly based on previous methods: Please refer to in solution digestion protocol: doi: 10.3389/fmicb.2015.00103. Mass spectrometer parameter settings: doi: 10.1074/mcp.O113.028787
Resource availability	The dataset used in this publication is part of a larger dataset freely accessibly on PRIDE PXD019140 MaxQuant Perseus

Introductory remarks

The proteome of the model organism *Escherichia coli* has been studied extensively and remains the best characterized amongst the prokaryotes [1–4]. Large scale bacterial proteomics have however traditionally been performed on *E.coli* cells grown in liquid cultures and despite considerable effort, these experiments have largely failed to identify virulence factors such as proteins associated with mobility, adhesion, response to specific challenges (including desiccation or aerobic stress) or thought to be important determinants in establishing bacterial infections. Indeed, until recently, proteomic analysis on clinical *E.coli* isolates grown in liquid culture failed to identify detectable flagellar proteins, which has been rationalised in terms of these isolates frequently being non-motile [5]: notably, several of these 'non-motile' *E.coli* isolates from the Keio collection infact possess motility genes, but appear to have lost their ability to activate those genes under culture conditions in which motility is necessary, perhaps as a result of accumulated mutations during growth and passaging in liquid culture [6]. Notably, it also seems likely that many important determinants associated with bacterial virulence and host cell adhesion are exclusively expressed during growth in biofilms, which can be crudely mimicked by colonies grown on solid media.

Significant progress has been made recently towards single-colony proteomics analysis, based on technological advances in mass spectrometry-based proteomics workflows. Such methods remove the need for liquid culture growth to produce sufficient biomass for analysis, thereby enabling exploration of the proteomes of minimally passaged clinical isolates of bacteria grown on primary culture plates and identification of both unique and differentially expressed proteins contained therein [7–9].

Here we present a simple workflow to characterize the unique proteome signature of a single colony forming unit using *E. coli* K12 strain grown on solid media [7,10].

Method details

Bacterial culturing and harvest of E.coli

The handling and culturing of the *E.coli* K12 strain should be performed in a Class-2 Biohazard safety cabinet (BSL-2). A freezer stock of *E.coli* K12 was inoculated into Lysogeny-Bertani (LB) media and cultured overnight to exponential growth phase. *E. coli* K12 cells from the fresh culture was

streaked onto LB agar plates and incubated at 37°C overnight. Six single colony forming units were picked separately from the solid LB agar media using a sterile glass pipettes (Thermo Fisher Scientific). Each single colony was handled separately and transferred to a sterile 1.5 ml Eppendorf tube containing filter sterilised Millipore grade water. The *E.coli* K12 cells from each single colony forming unit were washed by brief vortexing and the suspension of cells were harvested by centrifugation at 2000 g for 1 minute. The cells were washed once more and the harvested cell pellets were stored at -80°C until proteins were extracted.

Cell lysis and tryptic digestion

The frozen cell pellets were resuspended in Lysis buffer (10 mM Tris, Protease inhibitor cocktail tablets (Roche, Mannheim Germany and lysozyme 5 µg/ml (pH 6.8)) and incubated on ice for 30 minutes with gentle shaking. The proteins were then precipitated with equal volume of sample with methanol:chloroform (1v:0.75v). The collected precipitates containing proteins were resuspended in denaturation buffer (10 mM Tris, 6 M Urea, 2 M Thiourea (pH 8)). The precipitated proteins were quantified with the modified Bradford protein quantification method [11] by incorporating 1% HCL solution, Bradford Reagent and Bovine Serum Albumin (BSA) as a standard. A minimum of 20ug of protein for each single colony were handled separately and used to perform an efficient in-solution digestion on each lysate. The proteins were denatured with 1 mM dithiothreitol (DTT) for 1 hour at room temperature with gentle agitation and alkylated for 1 hour in the dark with 5.5 mM iodoacetamide (IAA). The diluted sample was then digested overnight with Trypsin (1:100 ratio) and digestion quenched with Trifluoroacetic acid (TFA) (Sigma Aldrich, St Louis, USA). Peptide preparations were then desalted using C18 stage tips [9] and dried before resuspension in 0.1% formic acid (FA) and 2% acetonitrile (ACN).

Mass spectrometry

Liquid chromatography separation was done with an in-house-packed precolumn (100 μM ID $\times$ 20 mm) connected to a 75 μM $\times$ 500 mm analytical column packed with C18 Luna beads (5 μm diameter, 100 Å pore size; Phenomenex 04A-5452). The columns were connected to an Ultimate 3500 RS nano UPLC system (Dionex). Two hundred nanograms of desalted peptides was loaded onto the column with starting mobile phase of 2% ACN, 0.1% FA. Peptides were eluted with the following gradient of 10 min at 2% ACN, increase to 25% ACN for 115 min, to 35% ACN over 5 min, to 80% ACN over 5 min, followed by a column wash of 85% for 20 min. The flow rate was constant at 300 μL/min. Typical back pressure values during separation were $<$350 bar. Mass spectra were acquired with an Q Exactive Orbitrap mass spectrometer in a data-dependent manner, with automatic switching between MS and MS/MS scans using a top-10 method. MS spectra were acquired at a resolution of 70 000 with a target value of 3×10^6 ions or a maximum integration time of 250 ms. The scan range was limited from 300 to 1750 m/z. Peptide fragmentation was performed via higher-energy collision dissociation (HCD) with the energy set at 28 NCE. Intensity threshold for ions selection was set at 1.3×10^4 with charge exclusion of $z = 1$ and $z > 5$. The MS/MS spectra were acquired at a resolution of 17 500, with a target value of 3×10^5 ions or a maximum integration time of 250 ms and the isolation window was set at 2.0 m/z.

Data processing, protein identification and data handling

The raw files for the 6 single colonies were analysed using MaxQuant Suite version 1.6.10.43 and the proteins were quantified using the LFQ intensities, MaxLFQ, in the MaxQuant protein groups output file.

Statistical analysis was performed by uploading the protein groups output file in the Perseus (1.6.10.45) module. Protein identifications were filtered to only consider proteins that had been identified with at least 2 unique peptides. Reverse contaminants were also filtered out together with potential contaminants. Initial summary statistics were obtained to assess the quality of the data. To validate the efficient protein extraction from single colonies for whole proteome analysis,

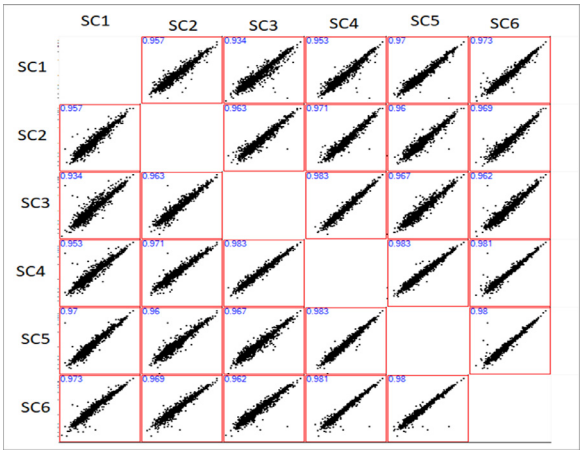

Fig. 1. Analysis of reproducibility between paired replicates. The multi scatter plot shows the log 2-transformed LFQ -values for protein groups identified in all six single colonies. The Pearson correlation coefficient is shown in each paired comparison and displayed in blue.

Table 1
Summary of number of proteins per single colony.

Single colony (SC)	Proteins per SC	Unique proteins in each SC
SC1	1667 (37%)	29
SC2	1558 (35%)	11
SC3	1635 (37%)	11
SC4	1704 (39%)	42
SC5	1424 (32%)	76
SC6	1521 (35%)	177

we performed the analysis on 6 biological replicates of *E. coli* single colonies. We plotted the log2-transformed iBAQ values of all 6 biological replicates of single colonies against each other using a multi scatter plot and calculated the Pearson correlation coefficient for each of the comparisons (Fig. 1). The protein extraction procedure is highly reproducible for all six single colonies, with Pearson correlation coefficient ranging between 0.934 – 0.983.

We defined proteins as present if at least two or more peptides per protein group and a q-value $<$0.01. Using the method described here, we identified total of 1769 proteins across all six single colonies and an overlap of 70% of proteins (Fig. 3). In Table 1 we represent the number of proteins identified in each single colony and we identified approximately 40% of the total proteome across these six single colonies. The overlap of protein identifications is described in Fig. 2 and summarized in Table 1.

Discussion

In proteomics, sample preparation procedure is a key component of experimental design since it strongly influences the sensitivity, accuracy of reproducibility of the downstream biological analysis and interpretation [12]. Here we present a simple protein extraction procedure from single colony forming units that can be used to characterize the proteomes of a wide variety of bacterial cells and optimized for different kinds of biological samples with a limiting amount of total extracted protein. In our proteomic analysis on individual isolated single colonies, we identified approximately 40% of the theoretical *E. coli* proteome across the six single colonies. The method described here thus provides a unique opportunity to explore the proteomes of minimally passaged isolates from primary culture plates and thereby potentially enables the direct identification of virulent and

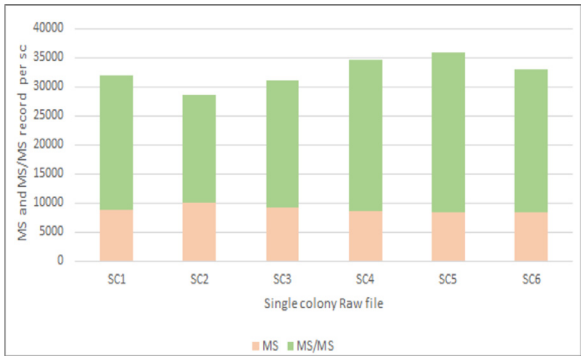

Fig. 2. Stacked bar graph showing the total MS and MS/MS recorded per Raw file of each single colony (SC). Orange bars showing the total MS recorded per SC, and their corresponding MS/MS indicated by stacked green bars. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

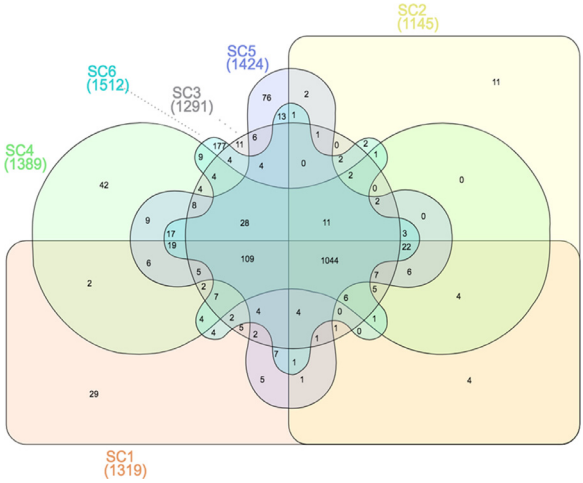

Fig. 3. Venn diagram of proteins across the six single colonies (SC1-6) demonstrating the overlap in number of protein between single colonies as well as the unique proteins identified in each single colony.

pathogenic bacteria in clinical isolates. In this proteomics experiment we identified amongst others key proteins uniquely expressed in *E. coli* cells grown on solid media that are implicated in swarming motility, influencing the spreading of bacterial cells on a surface (Supplementary Table 1), providing a unique insight into the differential expression of key virulence proteins within biofilm-like microenvironments in single colonies.

Data availability

Mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD019140

Declaration of Competing Interest

The authors of this article declare they have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101277.

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