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Analysis of the surface, secreted, and intracellular proteome of *Propionibacterium acnes*

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ABSTRACT

Propionibacterium acnes, plays an important role in acne vulgaris and other diseases. However, understanding of the exact mechanisms of *P. acnes* pathogenesis is limited. Few studies have investigated its proteome, which is essential for vaccine development. Here, we comprehensively investigate the proteome of *P. acnes* strain ATCC 6919, including secreted, cell wall, membrane, and cytosolic fractions in three types of growth media. A total of 531 proteins were quantified using an Orbitrap mass spectrometer and bioinformatically categorized for localization and function. Several, including PPA1939, a highly expressed surface and secreted protein, were identified as potential vaccine candidates.

1. Introduction

Acne vulgaris is an inflammatory disease of the pilosebaceous unit. With at least 85% prevalence among 12–24 year olds, it can cause long-term scarring and have a major psychological impact on individuals [1]. The gram-positive, anaerobic species *Propionibacterium acnes* has been traditionally implicated in the development of acne vulgaris [2]. It is also a significant organism in infections of the prostate [3], prosthetic joints [4], other surgical implants [5], spinal discs [6], and ophthalmic infections [7]. Unfortunately, the bacteria in many cases are resistant to antibiotic therapy [8,9], and other treatments often have low patient compliance [10]. Thus, the need exists for novel approaches to develop treatments that are more effective against *P. acnes* and have fewer side effects.

A vaccine may be an efficient means to protect against multiple types of infections caused by *P. acnes* [11,12]. Several recent studies have investigated this possibility. Antibodies generated by mouse intranasal vaccination of heat-killed *P. acnes* reduced *P. acnes*-induced IL-8 inflammation and cytotoxicity in sebocytes [13], though this study used an *in vitro* experimental system. Vaccination with heat-killed *P. acnes* reduced the severity of disease and inflammation in a *P. acnes* ear infection mouse model. The same group generated antibodies against *P. acnes* surface sialidase [14], which had similar effects in sebocytes. Vaccination with the sialidase [14], as well as with Christie–Atkins–Munch-Peterson (CAMP) factor 2 [15,16], also successfully reduced inflammation in mouse ear infections. However, this mouse model is not necessarily representative of the environment in acne vulgaris, in which an inflammatory response may act to either quickly clear *P. acnes*, or worsen the disease state. A better *in vivo* model is needed to further investigate vaccines.

In addition to its potential protective effects against acne, a *P. acnes*-based vaccine may also have other beneficial effects. Heat-killed *P. acnes* reduced atopic dermatitis in a mouse model, and increased the number of Th1 and Treg cells in the spleen [17]. A heat-killed *P. acnes* vaccine was also cross-protective against *Actinobacillus pleuropneumoniae* infection in mouse and pig models, inducing cross-reactive antibodies [18]. Specific *P. acnes* proteins could induce cross-reactive antibodies and protection in the *A. pleuropneumoniae* mouse model [19]. Intratumoral injection of live *P. acnes* was successful in increasing the antitumor Th1 immune response in a melanoma mouse model [20]. A *P. acnes* vaccine improved glomerulosclerosis in a mouse model via a Th1 response [21]. Several vaccine studies have also utilized *P. acnes* as an adjuvant. A microparticle preparation of *P. acnes* cell wall increased Th1 response to vaccination [22], and heat-killed P. acnes increased activation of B-1 lymphocytes [23].

P. acnes remains a largely understudied organism, with little information available to investigate additional vaccine candidates. Only four studies have covered the *P. acnes* proteome, none of which were comprehensive. Holland et al. examined the secreted

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proteome of several types of *P. acnes*, discovering interesting differences between types [24]. Dekio et al. identified several proteins expressed by *P. acnes* in anaerobic and microaerophilic conditions, but not aerobic conditions [25]. Mak et al. assessed the surface proteome of *P. acnes* using trypsin shaving, comparing it to other *Propionibacterium* species [26]. Bek-Thomsen et al. examined the proteome of sebaceous follicular casts, which included several *P. acnes* proteins [27]. However, none of these studies used a quantitative method, and only a limited number of proteins could be detected. A more comprehensive picture of its proteome, including proteins from all fractions of the cell, may contribute to our understanding of the molecular mechanisms of *P. acnes* disease pathogenesis, in addition to suggesting additional vaccine candidates. Here, we present a comprehensive study of the proteome, including surface proteins, secreted proteins, and intracellular proteins, of *P. acnes* strain ATCC 6919 (phylotype IA-2, a group enriched in acne vulgaris patients [28,29]) grown in three types of media.

2. Methods

2.1. Bacterial culture

P. acnes strain ATCC 6919 (NCTC 737), a commonly used laboratory strain originally isolated from an acne patient, was inoculated from glycerol stocks into 10 mL of Reinforced Clostridial Media (RCM) (Oxoid) and grown at 37 °C using AnaeroPack system sachets (Remel). When bacteria reached the exponential phase of growth (optical density of 0.1–0.3 at 600 nm wavelength with 1 cm path length) after 5–6 days, bacteria were collected by centrifugation and divided evenly into 50 mL of RCM, 50 mL of Brain–Heart Infusion Broth (BHI) (Oxoid), and 50 mL of BHI supplemented with 5% egg yolk (Sigma) after autoclaving (EBHI). Cultures were again incubated at 37 °C for approximately 40 h using anaerobic sachets, with shaking at 200 rpm for the cultures in BHI and EBHI. *P. acnes* was harvested in the late exponential phase (optical density of ~1.0 at 600 nm wavelength with 1 cm path length) for protein fraction preparation.

2.2. Fraction preparation

P. acnes samples were pelleted by centrifugation at 4000 g for 10 min for the BHI and BHI-E samples and 30 min for the RCM sample. The supernatant, containing the secreted proteins, was collected and filtered through 0.2 μm pores, yielding the Cell Secretion (CS) fraction. The pellets were washed thrice with phosphate buffered saline (PBS), and divided into four equally sized samples.

To protoplast the bacteria and release cell wall proteins, a technique was followed similar to one used by Gallis et al. [30]. One sample was resuspended in 200 μL of solution containing 10 mM pH 7 phosphate buffer, 600 mM KCl, 10 mM $MgCl_2$, and 1 mg/mL egg white lysozyme (Pierce). Another sample was resuspended in 200 μL of solution containing 50 mM Tris–HCl, 250 mM sucrose, 10 mM $MgCl_2$, 30 mM KCl, and 1 mg/mL egg white lysozyme. These two samples were incubated with rotation at 37 °C for 4 h to allow for lysozyme digestion of cell walls. Samples were then centrifuged for 5 min at 1000 g with the supernatant retained. Samples were than centrifuged for 5 min at 20,000 g, and supernatants from the two fractions were combined. The sample was filtered through 0.2 μm pores, yielding the Cell Wall (CW) fraction.

The remaining two samples were subjected to beadbeating with a micro-MiniBeadbeater (Biospec Products) for five minutes with cooling every minute. Samples were than sonicated. One of these samples was designated the Total Cell Extract (TCE) fraction. The other sample was centrifuged at first 1000 g for 5 min and then at 8000 g for five minutes, with the supernatant retained each time. The sample was then centrifuged at 20,000 g for 15 min. The supernatant was designated the Cell Cytosolic (CC) fraction. Membranes were obtained using a similar protocol to one developed by Zuobi-Hasona and Brady [31]. The pellet was washed three times with PBS with centrifugation at 20,000 g for 15 min each, and it was than resuspended in 100 μL of PBS. This was designated the Cell Membrane (CM) fraction.

2.3. Mass spectrometry

10 μg of protein from the CC and TCE fractions as quantified by Bradford assay and all of the CS, CW, and CM protein samples were adjusted to 20% trichloroacetic acid and incubated at 4 °C for 30 min. Samples were centrifuged at 20,000 g for 5 min, and the pellet was washed with 200 μL of cold acetone. The pellet was resuspended in a solution of 50% aqueous 100 mM ammonium bicarbonate and 50% acetonitrile. Samples were reduced with 25 mM tris-(2-carboxyethyl)-phosphine for 30 min at 37 °C and then alkylated with 75 mM iodoacetamide for 1 h at room temperature in the dark. Samples were diluted to 5% acetonitrile in pH 8100 mM ammonium bicarbonate buffer and digested for 16 h with 500 ng trypsin (Promega). Samples were then centrifuged at 20,000 g for 10 min twice, with the supernatant retained. Samples underwent liquid chromatography and tandem mass spectrometry using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher) with NanoLC-2D HPLC (Eksigent). This method has been shown to be accurate for label-free quantification of proteins [32,33]. Reverse phase chromatography on a reverse phase column (New Objective C18, 15 m, 75 μM diameter) was conducted at 500 nL/min for loading and analytical separation with Buffer A containing aqueous 0.1% formic acid and Buffer B containing 0.1% formic acid in acetonitrile. Peptides were eluted using a gradient of 3–40% Buffer B over 3 hours. The Orbitrap was used in MS/MS mode with a high-resolution full precursor scan and ten low resolution MS/MS events on the linear trap during the full scan., The threshold intensity for Collision Induced Dissociation was 5000 and the allowed mass range was 350–2000 Da.

2.4. Data analysis

Raw spectral data were processed with RawXtract, and identified peptides were analyzed with the ProLuCID algorithm (V1.3.3) using the database of *P. acnes* reference strain KPA171202 [34]. No additional unique protein hits were found when using the type IA *P. acnes* strain 266 database. Scaffold 4.4.1.1 (Proteome Software) was used for identification and quantification of proteins with a false discovery rate of 5%, allowing for more true identifications in the smaller CS and CW datasets than the standard 1%. Only proteins with at least two unique peptides were counted. Protein quantification was determined by normalized spectral abundance factors to obtain relative quantification of protein in each sample. All quantities are reported in fmol protein per microgram of total protein detected. Protein localization was predicted using the PSORTb 3.0 tool [35], and signal peptides were predicted using SignalP 4.1 [36]. PSORTb assigns scores for extracellular, cell wall, membrane, or cytoplasmic localization, with the sum adding up to 10. A “non-cytoplasmic” PSORTb localization indicates a zero score for cytoplasmic localization, while “unknown” indicates no localization prediction. In Table S1, only the highest score for any fraction is shown. For SignalP, a score of over 0.45 was indicative of a signal peptide (Y), with scores of 0.35–0.45 listed as probable signal peptides (P), scores of 0.25–0.35 listed as maybe a signal peptide (M), and scores of

below 0.25 listed as no signal peptide (N). NCBI's BLAST tool [37] was used to search for homologs with known function to assist with functional annotation of protein lists.

3. Results

3.1. Identification of Propionibacterium acnes proteins

To investigate the proteome of *P. acnes* in different environments, we assessed its proteome in Reinforced Clostridial Media (RCM) and Brain–Heart Infusion Broth (BHI), common media used in laboratories. Additionally, we devised EBHI media, BHI supplemented with 5% egg yolk, to approximate the lipid-rich environment of the pilosebacous unit. In total, 531 proteins from *P. acnes* were identified and quantified (Table S1), representing slightly over 20% of the total number of predicted proteins in its genome. Of these, 471 were detected in the fractions from *P. acnes* grown in Reinforced Clostridial Media RCM, 359 in fractions from BHI, and 415 in fractions from EBHI. Most proteins were found in all three growth media groups, including nearly all from BHI fractions (Fig. 1A).

Cell Secretion (CS) fractions together across the three different media contained 58 distinct proteins (Fig. 1B). However, very few proteins were detected in CS fractions from BHI and EBHI media due to a large amount of precipitate after treatment with trichloroacetic acid that could not be separated from protein. Cell Wall (CW) fractions contained 123 distinct proteins across the three different media. Cell Membrane (CM) fractions contained 310 proteins, though the BHI CM fraction had only 34, significantly fewer than the RCM and EBHI fractions. Cell Cytosolic (CC) fractions contained 445 proteins. All fractions had significant overlap of proteins with other fractions, but few proteins were present in all four of these fractions (Fig. 1B). Additionally, the Total Cell Extract (TCE) fraction contained 347 proteins, of which only 5 were unique to this fraction. The TCE fraction had a very high degree of overlap with the CC fraction.

3.2. Protein localization

To assess the purity of our protein fractions, we utilized PSORTb to predict protein localization and SignalP to predict signal peptides, which are indicative of a non-cytoplasmic localization. Both PSORTb and SignalP were in fairly good agreement for non-cytoplasmic localization, with SignalP more stringent in its assessments. Of the PSORTb predicted cytoplasmic proteins, only 12 out of 384 proteins (3.1%) had a signal peptide or probable signal peptide. Of the PSORTb predicted non-cytoplasmic proteins (including predicted cell wall, extracellular, and membrane proteins), 35 out of 94 proteins (37.2%) had a signal peptide or probable signal peptide. For PSORTb unknown proteins, 17 out of 53 proteins (32.1%) had a signal peptide or probable signal peptide, indicating that the unknown proteins may contain nearly the same proportion of true non-cytoplasmic proteins and PSORTb predicted non-cytoplasmic proteins.

The CS fractions were highly enriched for non-cytoplasmic proteins according to PSORTb, with 31 out of 58 proteins (53.4%) having non-cytoplasmic (including unknown) localization prediction. In the 20 most abundant CS proteins, the level of enrichment was even higher (85%) (Table 1). The CW fractions were also enriched for non-cytoplasmic proteins, with 53 out of 123 proteins (43.1%) having non-cytoplasmic localization prediction. The level of enrichment was also higher (60%) for the 20 most abundant CW proteins (Table 2), not including a phosphocarrier protein that may have a signal peptide and act as a cell surface transporter. The CM fractions had 80 out of 310 proteins (25.8%) with non-cytoplasmic localization prediction. This is only slightly higher than the proportion in the CC fraction (22.7%). The proportion of non-cytoplasmic proteins was not further enriched in the most abundant CM proteins. In the CC fraction, all of the 20 most abundant proteins had cytoplasmic localization (Table 3).

3.3. Functional analysis

NCBI's BLAST was used to assess the function of proteins by homology. Similar proteins were identified, together with conserved domains, and this information was used to assess a possible function of 176 proteins (Table S1). In the CS fractions, 40% of the 30 most abundant proteins were associated with digestion of protein, lipid, or carbohydrate nutrients (Fig. 2A). Proteins of unknown function represented 20% of the CS proteins, and 16.7% were possible surface proteins (involved in cell wall remodeling, adhesion, transportation, and mobility) that had been released. The remainder in the CS fraction were cytosolic proteins of varying function. In the CW fractions, 36.7% of the most abundant 30 proteins were associated with varying cell surface-related activities including nutrient digestion, adhesion, transportation, cell wall remodeling, as well as one protein with putative antitoxin properties (Fig. 2B). Comparatively, 23.3% of the CS proteins were unknown, and the rest were cytosolic proteins, with ribosomal proteins particularly well-represented. The CC fraction was

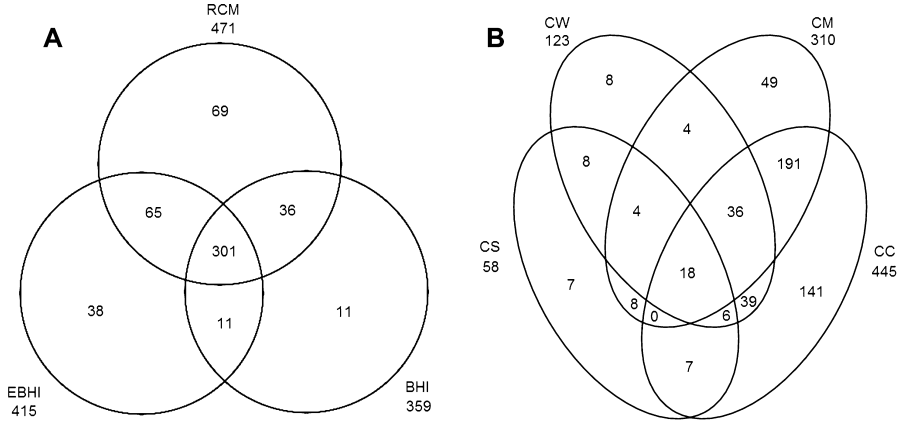

Fig. 1. Identified Proteins. Comparison of proteins identified in different growth media (A) and different cell fractions (B). Data from one of two similar experiments. Abbreviations: RCM—Reinforced Clostridial Media, BHI—Brain–Heart Infusion Broth, EBHI—5% Egg Yolk Supplemented Brain Heart Infusion Broth, CS—Cell Secretion, CW—Cell Wall, CM—Cell Membrane, CC—Cell Cytosolic.

Table 1
The 20 most abundant secreted proteins.

Protein	Accession (gi)	MW (kDa)	RCM fmol	BHI fmol	EBHI fmol	SignalP	PSORTb	Function
Protein PPA1939	50843388	17	6222	58824	6757	Y	Unknown	Unknown
Adhesion	50843565	42	50	0	19238	M	Unknown	Adhesion
cAMP factor	50842175	29	3408	0	2660	Y	Extracellular	Digestion
Protein PPA2239	50843674	41	1996	0	0	Y	Non-Cytoplasmic	Digestion
Protein PPA2271	50843708	52	1415	0	0	Y	Unknown	Digestion
Endoglycoceramidase	50842131	57	1351	0	0	Y	Non-Cytoplasmic	Digestion
Protein PPA1746	50843206	22	1218	0	0	Y	Non-Cytoplasmic	Unknown
NPL/P60 protein	50842209	41	1100	0	0	Y	Membrane	Digestion
Cell wall hydrolase	50843410	43	986	0	0	M	Extracellular	Digestion
Protein PPA1745	50843205	90	979	0	0	M	Extracellular	Digestion
cAMP factor	50842820	30	685	0	0	Y	Extracellular	Digestion
Chaperone GroEL	50841936	57	662	0	0	N	Cytoplasmic	Protein folding
Triacylglycerol lipase	50843543	36	642	0	0	Y	Non-Cytoplasmic	Digestion
Protein PPA0533	50842017	20	599	0	0	Y	Non-Cytoplasmic	Unknown
co-chaperonin GroES	50843233	11	595	0	0	N	Cytoplasmic	Protein folding
Endoglycoceramidase	50843544	54	571	0	0	Y	Non-Cytoplasmic	Digestion
Fine tangled pili	50843572	19	436	0	0	N	Cytoplasmic	Mobility
Lipase/acylhydrolase	50843480	30	377	0	0	P	Unknown	Digestion
Regulatory protein	50842205	39	325	0	0	P	Unknown	Translation
Protein PPA1715	50843175	49	309	0	0	Y	Non-Cytoplasmic	Unknown

Table shows quantity in femtomoles per microgram of total protein detected. SignalP predictions are yes (Y), probably (P), maybe (M), and no (N). Abbreviations: RCM—Reinforced Clostridial Media, BHI—Brain Heart Infusion Broth, EBHI—5% Egg Yolk Supplemented Brain Heart Infusion Broth. Data from one of two similar experiments.

dominated by proteins involved in metabolism, translation, and protein folding, with significant numbers of proteins involved in synthesis and DNA structure (Fig. 2C).

4. Discussion

By examining the proteome in three different types of growth media, we can gain insight into which proteins may be expressed in a wide variety of environments *in vivo*, including the pilosebaceous unit in acne and those environments in other *P. acnes* infections. RCM and BHI are common media used to culture *P. acnes in vivo*, but these do not provide a lipid-rich environment to approximate the pilosebacous gland. Thus, we investigated a third media, EBHI, which uses egg yolk as a source of lipids. Of note, a large number of proteins were expressed by *P. acnes* in all three types of media, indicating that its proteome is largely conserved between different environments. This may be due to the small ecological niche that *P. acnes* occupies, reducing its need for an ability to significantly change its proteome for different environments. Since most of those conserved proteins identified *in vitro* across media types would likely be expressed *in vivo*, our findings may also apply to the *P. acnes* proteome in the pilosebacous unit, and thus easing the identification of good vaccine candidates. Indeed, the proteins detected *in vivo* by Bek-Thomsen et al. were among the most abundant in our *in vitro* fractions [27]. Nevertheless, we did detect a few interesting differences between media types. In BHI media, we found lower expression of some adhesion proteins (50843565, 50843645) and lipases (50843205, 50843480, 50843543), perhaps reflective of the increased nutrient density and variety in EBHI and especially RCM. One CAMP factor was also not found in the CS and CW fractions of BHI, but it was abundant in RCM and EBHI.

The CS and CW fractions contained many proteins with "unknown" or "non-cytoplasmic" localization prediction according to PSORTb. It seems likely that the bulk of these proteins are true secreted proteins or cell wall proteins, implying that the PSROTb program can be further refined for greater predictive accuracy for these types of proteins. Also many proteins with predicted

Table 2
The 20 most abundant cell wall proteins.

Protein	Accession (gi)	MW (kDa)	RCM fmol	BHI fmol	EBHI fmol	SignalP	PSORTb	Functional group
co-chaperonin GroES	50843233	11	5024	251	2008	N	Cytoplasmic	Protein folding
Protein PPA1939	50843388	17	2799	1799	1555	Y	Unknown	Unknown
Membrane lipoprotein	50843218	34	91	4124	1924	M	Membrane	Transportation
Adhesion	50843565	42	2553	0	3283	M	Unknown	Adhesion
Protein PPA2271	50843708	52	800	1948	1539	Y	Unknown	Digestion
Protein PPA2334	50843769	126	0	4210	0	N	Cytoplasmic	Unknown
DNA-binding HU	50843144	10	1948	749	1427	N	Cytoplasmic	Miscellaneous
CsbD-like protein	50842907	7	2477	635	743	N	Unknown	Unknown
Protein PPA1281	50842762	30	1297	219	1754	M	Non-Cytoplasmic	Unknown
Peptide transporter	50843590	61	1311	0	1813	Y	Cell Wall	Transportation
Phosphocarrier HPr	50841838	9	1424	508	240	M	Cytoplasmic	Transportation
Protein PPA1018	50842501	7	1033	271	696	N	Unknown	Miscellaneous
Protein PPA1715	50843175	49	1265	384	96	Y	Non-Cytoplasmic	Unknown
Rare lipoprotein A	50843612	37	0	1553	113	Y	Non-Cytoplasmic	Cell wall structure
Protein PPA0542	50842026	18	510	0	960	Y	Non-Cytoplasmic	Cell unknown
50S ribosomal L29	50843310	9	1052	246	139	N	Cytoplasmic	Translation
Adhesion	50843645	48	45	0	1356	P	Cell Wall	Adhesion
tRNA synthetase	50842977	64	0	0	1369	N	Cytoplasmic	Synthesis
30S ribosomal S15	50842951	10	604	238	526	N	Cytoplasmic	Translation
30S ribosomal S18	50843663	9	155	333	766	M	Cytoplasmic	Translation

Table shows quantity in femtomoles per microgram of total protein detected. SignalP predictions are yes (Y), probably (P), maybe (M), and no (N). Abbreviations: RCM—Reinforced Clostridial Media, BHI—Brain Heart Infusion Broth, EBHI—5% Egg Yolk Supplemented Brain Heart Infusion Broth. Data from one of two similar experiments.

Table 3
The 20 most abundant cytoplasmic proteins.

Protein	Accession (gi)	MW (kDa)	RCM fmol	BHI fmol	EBHI fmol	SignalP	PSORTb	Functional group
GAPDH	50842303	36	943	1270	1013	N	Cytoplasmic	Metabolism
co-chaperonin GroES	50843233	11	1206	1108	605	N	Cytoplasmic	Protein folding
Phosphopyruvate hydratase	50842029	46	680	1193	699	N	Cytoplasmic	Metabolism
50S ribosomal L7/L12	50843339	14	546	816	695	N	Cytoplasmic	Translation
Elongation factor Tu	50843327	44	649	795	480	N	Cytoplasmic	Translation
Fructose-bisphosphate aldolase	50843457	37	430	605	681	N	Cytoplasmic	Metabolism
Phosphoglyceromutase	50841849	28	519	573	523	N	Cytoplasmic	Metabolism
Aspartate aminotransferase	50841624	39	443	478	568	N	Cytoplasmic	Synthesis
Molecular chaperone GroEL	50843232	56	755	368	349	N	Cytoplasmic	Protein folding
DNA-binding protein HU	50843144	10	351	539	517	N	Cytoplasmic	DNA structure
Molecular chaperone GroEL	50841936	57	637	261	382	N	Cytoplasmic	Protein folding
Phosphoglycerate kinase	50842304	42	262	476	456	N	Cytoplasmic	Metabolism
Malate dehydrogenase	50843200	35	367	298	427	N	Cytoplasmic	Metabolism
Molecular chaperone DnaK	50843484	66	379	265	430	M	Cytoplasmic	Protein folding
Elongation factor Ts	50842998	28	249	374	378	N	Cytoplasmic	Translation
Hypothetical protein PPA0277	50841766	12	45	555	311	N	Cytoplasmic	DNA structure
Polynucleotide phosphorylase	50842950	79	225	301	356	N	Cytoplasmic	Miscellaneous
Fine tangled pili	50843572	19	381	189	282	N	Cytoplasmic	Mobility
Ornithine carbamoyltransferase	50842070	37	386	179	234	N	Cytoplasmic	Synthesis
Triosephosphate isomerase	50842305	27	213	241	330	N	Cytoplasmic	Metabolism

Table shows quantity in femtomoles per microgram of total protein detected. SignalP predictions are yes (Y), probably (P), maybe (M), and no (N). Abbreviations: RCM—Reinforced Clostridial Media, BHI—Brain Heart Infusion Broth, EBHI—5% Egg Yolk Supplemented Brain Heart Infusion Broth. Data from one of two similar experiments.

membrane localization were detected in the CW fractions, including several transporters. While lysozyme digestion of the cell wall should have left the membrane intact, it is possible that some membrane proteins may have components embedded in the cell wall that were released upon cell wall disruption.

There were several proteins of predicted cytoplasmic localization in our CS and CW fractions, several of which have been seen in previous studies [24,26]. While some of these are likely contaminants, natural autolysis of *P. acnes* could result in many of these proteins being released into the media and later binding to the surface of the bacteria. Additionally, these proteins may have dual roles outside of the cytoplasm, and may be secreted by non-traditional pathways, accounting for their cytoplasmic localization prediction. Furthermore, many abundant CC proteins were not found in CS and CW fractions, indicating that those possible contaminating proteins represent only a subfraction of likely contaminants and thus, are largely not contaminants at all. Finally, functional analysis of CS and CW proteins indicates that these prediction programs are likely accurate, since they predicted that proteins with expected secreted and cell wall functions were non-cytoplasmic.

More refined methods are needed to obtain pure CM fractions of *P. acnes*. Even protocols utilizing ultracentrifugation yielded no improvement in purity. However, it should be noted that while the CC and TCE fractions have a very high degree of overlap, the CM fraction has a fair number of unique protein identifications in comparison, implying that this protein fraction is distinctly different from a purely cytosolic fraction, which would be the case if contaminants completely dominated the CM fraction.

The RCM CS fraction shared many highly expressed proteins in common with a study on the *P. acnes* secretome by Holland et al. [24]. While not directly comparable due to the different type of media used, the fact that similar highly expressed proteins were found in both studies and in a separate *in vivo* study [27] supports both methods used. While Holland et al.'s method of 2D gel digestion allowed for use of BHI, and their earlier exponential phase cultures allowed for higher purity (fewer cytosolic proteins, likely due to less autolysis), our use of in-solution digestion followed by assessment with an Orbitrap mass spectrometer allowed for many more proteins to be both identified and quantified. These same conclusions apply to the study of the cell wall proteome by Mak et al. [26], where many of the same highly expressed *P. acnes* proteins were also detected in both studies.

With regard the existing vaccine candidates, the most highly expressed CAMP factor (50842175) detected in our fractions was the same protein used in a vaccine to reduce inflammation in a

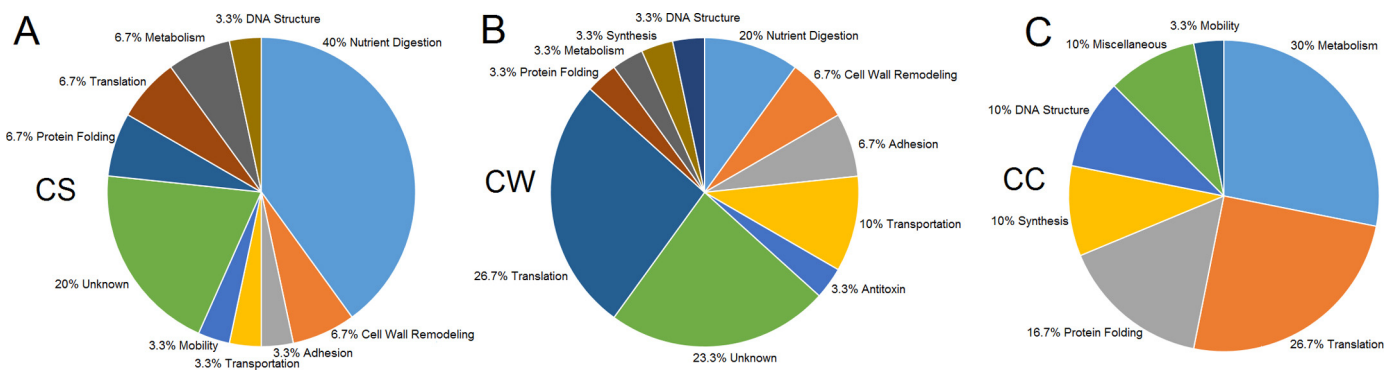

Fig. 2. Protein Functional Analysis. Functional analysis of the 30 most abundant proteins in the secreted fraction (A), cell wall fraction (B), and cytosolic fraction (C). Abbreviations: CS—Cell Secretion, CW—Cell Wall, CC—Cell Cytosolic.

mouse ear infection model [16]. In contrast, the surface sialidase, which conferred similar vaccine protection [14], was not detected in our protein fractions or in the *in vivo* study of Bek-Thomsen et al. [27]. The CAMP factor protein may therefore have a higher probability of *in vivo* expression in the pilosebaceous environment. Several proteins from *P. acnes* were found to confer vaccine protection in mice against *A. pleuropneumoniae* [19]. The most efficient of these was a single-stand DNA binding protein (50843664), which was detected at moderate level in our CC fraction. Phage shock protein A (50842186), the second-most efficient vaccine in the mouse model, was detected at a somewhat lower level in the CC fraction. All other proteins in their study were also detected in our CC fraction in varying quantity, indicating that they may be also be used in a *P. acnes*-based vaccine as well.

Several highly expressed surface and secreted proteins detected in our study are of potential future interest for functional studies or vaccine candidates. These include the PPA1939 protein (50843388) of unknown function, which was the most abundant secreted protein, and among the most abundant in the cell wall. Other surface proteins that are highly expressed and may make good vaccine candidates include DsA1, an adhesion/S-layer protein (50843565), and a probable transporter lipoprotein (50843218). Since *P. acnes* is a commensal organism and acne is an inflammatory disease, it may be preferable for an acne vaccine candidate to induce a strong Treg response, rather than the more common Th1, Th17, or antibody response. Thus, several vaccine candidates would need thorough assessment using human cells to find one suitable for use in acne. These same highly expressed surface and secreted proteins may be potential virulence factors. Further research to determine their function may allow for greater understanding of the mechanisms of pathogenesis in acne.

We investigated *P. acnes* strain ATCC 6919, a MLST phylotype IA_1 [9,29] and whole-genome phylotype IA-2 [38] strain of *P. acnes*. Many other phylotypes are of potential interest, since they have been recently shown to have differing disease associations, including for acne vulgaris [28,29,39]. We are currently characterizing the full proteome of several of these phylotypes.

5. Conclusions

Our study presents a comprehensive overview of the *P. acnes* proteome, with proteins identified and quantified using an Orbitrap mass spectrometer. In addition to cytoplasmic proteins, we also identified several dozen secreted and cell wall proteins, which were analyzed for predicted localization and function. Our identified cell wall proteins, due to their surface localization, represent potential vaccine candidates.

Conflict of interest

JK has consulted for Allergen, Leo Pharma, Anacor, and TPG.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.euprot.2015.06.003.

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