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# Passive immunoprotection targeting a secreted CAMP factor of *Propionibacterium acnes* as a novel immunotherapeutic for acne vulgaris

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ABSTRACT

*Propionibacterium acnes* (*P. acnes*) bacteria play a key role in the pathogenesis of acne vulgaris. Although our previous studies have demonstrated that vaccines targeting a surface sialidase or bacterial particles exhibit a preventive effect against *P. acnes*, the lack of therapeutic activities and incapability of neutralizing secretory virulence factors motivate us to generate novel immunotherapeutics. In this study, we develop an immunotherapeutic antibody to secretory Christie–Atkins–Munch-Peterson (CAMP) factor of *P. acnes*. Via agroinfiltration, *P. acnes* CAMP factor was encapsulated into the leaves of radishes. ICR mice intranasally immunized with whole leaves expressing CAMP factor successfully produced neutralizing antibodies that efficiently attenuated *P. acnes*-induced ear swelling and production of macrophage-inflammatory protein-2. Passive neutralization of CAMP factor enhanced immunity to eradicate *P. acnes* at the infection site without influencing bacterial growth elsewhere. We propose that CAMP factor is a novel therapeutic target for the treatment of various *P. acnes*-associated diseases and highlight the concept of neutralizing *P. acnes* virulence without disturbing the bacterial commensalism in human micorbiome.


## 1. Introduction

*Propionibacterium acnes* (*P. acnes*) is a Gram-positive, anaerobic, ubiquitous commensal, and opportunistic pathogen [1,2]. Nearly everyone hosts *P. acnes* [3,4], which accounts for approximately half of the total skin microbiome [5], with an estimated density of $10^2$–$10^{5-6}$ $cm^2$ [6,7]. *P. acnes* predominates (more than 46% of total bacteria) in facial skin [8]; however, it can be found almost everywhere on the body [9,10]. *P. acnes* colonizes the sebaceous follicles [6] and is one of the pathogens involved in the *progression* of inflammation in *acne* vulgaris [11,12] and tissue damage by releasing various virulence factors [13,14]. The inflammatory reaction is marked initially by suppuration, followed by granulomatous inflammation, and, over time, by fibrosis and scarring. Once the hair follicle wall has ruptured, *P. acnes* escapes from the damaged follicles and then enters the dermis in most cases of late-stage and/or severe acne vulgaris [15]. Aside from acne *vulgaris*, many human diseases such as implant infections, pulmonary sarcoidosis, osteomyelitis and endocarditis have been linked to *P. acnes* infections [9,16,17].

Examination of the genome of *P. acnes* has revealed that Christie–Atkins–Munch-Peterson (CAMP) factor is a potential secretory virulence factor [18]. The bacterium carries five genes with sequence homology (approximately 32%) to the co-hemolytic CAMP factor of *Streptococcus agalactiae* (*S. agalactiae*) [19,20]. CAMP factor of *S. agalactiae* potentially can bind to the $F_c$ fragment of immunoglobulins of the Immunoglobulin G (IgG) and Immunoglobulin M (IgM) classes [19]. In addition, it has been reported that CAMP factor of *S. agalactiae* acts as a pore-forming toxin [20]. Although it is unclear if *P. acnes* CAMP factor exhibits a similar co-hemolytic activity as that of *S. agalactiae*, it has been reported that when *P. acnes* was grown on a sheep blood agar plate in close proximity to β-hemolytic microorganisms [21], it synergistically enhances hemolysis similar to the classical CAMP reaction

*Abbreviations:* ATCC, American Type Culture Collection; CAMP factor, Christie, Atkins, Munch-Peterson factor; CFU, Colony forming unit; $ddH_2O$, *Didistilled* water; *E. coli*, *Escherichia coli*; ELISA, Enzyme-linked immunosorbent assay; GFP, Green fluorescence protein; GUS, β-Glucuronidase; His, Histidine; H&E, Hematoxylin and eosin; ICR, Institute of Cancer Research; IgG, Immunoglobulin G; IgM, Immunoglobulin M; IL, Interleukin; IPTG, Isopropyl-β-D-thiogalactoside; LB, Luria-Bertani; MBP, Maltose binding protein; MIP-2, Macrophage-inflammatory protein-2; OD, Optical density; PBS, Phosphate-buffered saline; *P. acnes*, *Propionibacterium acnes*; PCR, Polymerase chain reaction; PTMs, posttranslational modifications; RCM, Reinforced clostridium medium; SCAP, Spore coat-associated protein; SDS–PAGE, Sodium dodecyl sulfate-polyacrylamide gel; *S. agalactiae*, *Streptococcus agalactiae*; *S. aureus*, *Staphylococcus aureus*; TNF-α, tumor necrosis factor-α; UV, Ultraviolet.

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first described by Charlistie and co-workers [22]. Moreover, we have recently demonstrated that *P. acnes* CAMP factor enhances hemolysis and cytolysis by *Staphylococcus aureus* (*S. aureus*) β-hemolysin, suggesting that *S. aureus* may shrewdly utilize the secreted *P. acnes* CAMP factor to intensify its virulence [23].

There are many challenges in treating acne *vulgaris*. Current treatments using anti-acne agents including antibiotics lack bacterial specificity, imbalance human microbiome homeostasis, and have a risk of generating drug-resistant bacteria [24]. Benzoyl peroxide, an agent for mild acne, releases oxygen free radicals that oxidize bacterial proteins in the sebaceous follicles to decrease the number of anaerobic bacteria and irritating-type free fatty acids [25]. Although its use does not predispose to skin infection and develop bacterial resistance [26], it has some adverse effects on the skin that may include stinging, dryness, and peeling [27]. The increased oxygen free radical by benzoyl peroxide could even increase the risk of skin cancer [25,28]. Importantly, most antibiotics targeting bacterial particles are incapable of inactivating the secretory toxins [29]. Alternatively, isotretinoin is a powerful and effective medication derived from vitamin A [30], often prescribed by doctors to treat severe acne only after other treatments have failed. However, isotretinoin is strictly regulated due to the induction of serious side effects. As little as one dose of isotretinoin can cause severe birth defects in pregnant woman taking this medicine [31]. *P. acnes* has been recognized as a ubiquitous commensal on the human body [32,33] and only becomes pathogenic in some diseases [13,34]. Systemic treatment of *P. acnes* infection using anti-acne agents or antibiotics may carry risks of disrupting the commensalisms of *P. acnes* and have incapacity to naturalizing secretory toxins of *P. acnes.*

In our previous efforts, we have generated anti-*P. acnes* vaccines using a surface sialidase [35] and killed *P. acnes* [12] as antigens. Although we have demonstrated that these anti-*P. acnes* vaccines decrease *P. acnes*-induced inflammation [35], they may not have the capability to neutralize the virulence factors secreted from *P. acnes*. In addition, these vaccines designed as preventive modalities may lack the therapeutic effects. Notably, to achieve preventive effects, these anti-*P. acnes* vaccines have to be administrated in the early childhood. Many people may be reluctant to receive these vaccines since they cannot predict if they will suffer from acne *vulgaris*. Thus, there is an urgent need for the development of immunotherapeutics for acne *vulgaris*. It has been documented that inhibition of secreted virulence factors may present less selective pressure for the generation of microbial resistance [36]. Inhibition of secreted virulence factors may not directly influence the growth of commensal *P. acnes* [37], minimizing the risk of altering the homeostasis of resident human microbes. Accordingly, neutralization of bacteria-induced virulence and inflammation without directly killing bacteria would be an excellent immunotherapeutic for the treatment of acne *vulgaris*. After neutralization of secreted virulence factors, the "disarmed" bacteria in local lesions could be eliminated naturally by immune systems. Therefore, passive transfer of antibodies against toxins would complement other treatments, as it would be able to neutralize circulating *P. acnes* toxins while keeping the *P. acne* at an optimal balance. Thus, passive immunization to toxins of *P. acnes* in place of commonly used therapy such as anti-acne agents and antibiotics would have benefit for certain condition of skin inflammation.

In this study, we employ a passive immunization approach to attenuate the virulence of secretory CAMP factor of *P. acnes*. The factor was expressed in plant leaves using agroinfiltration. There are several advantages to expressing the proteins in the plants, such as low cost and high yield [38,39]. Plants can also be grown on site, reducing the need for costly refrigerated transport and storage [40,41]. Furthermore, the main advantages associated with plants include posttranslational modifications (PTMs) and production of correctly folded and assembled multimeric proteins, low risk of contamination with pathogens and endotoxins such as those occurring in mammalian and bacterial systems, and the avoidance of ethical problems associated with transgenic animals and animal materials [42].

Overall, this study provides a novel therapeutic target (CAMP factor) for treatment of acne *vulgaris* and presents a concept of suppressing *P. acnes*-induced local lesions without disturbing the commensalisms of *P. acnes*. The concept may be able to be broadly applied for treating human diseases caused by commensal microbes that become pathogens in local lesions [43].

## 2. Materials and methods

### 2.1. Molecular cloning and expression of recombinant green fluorescence protein (GFP) and CAMP factors

A polymerase chain reaction (PCR) product encoding a putative mature protein (29–267 amino acid residues) of CAMP factor (accession number: gi/50842175) was generated using gene-specific primers based on the complete genome of *P. acnes* [13]. The forward PCR primer (5′-TAAGGCCTCTGTCGACGTCGAGCCGACGACGACCATCTCG-3′) consisted of nucleotides containing a *Sal*I site (GTCGAC) and the reverse PCR primer (5′-CAGAATTCGCAAGCTTGGCAGCCTTCTTGACATCGGGGGAG-3′) consisted of nucleotides containing a *Hind*III site (AAGCTT). PCR was performed by using *P. acnes* genomic DNA as a template. The amplified DNA products were inserted at the restriction enzyme sites into an In-Fusion™ Ready pEcoli-6 × HN-GFPuv expression vector and transformed into competent cells [*Escherichia coli (E. coli)*, BL21 (DE3), Invitrogen, Carlsbad, CA, USA], which were subsequently selected on Luria-Bertani (LB) plates containing ampicillin (50 μg/ml) and cultured overnight at 37 °C. To express the GFP and CAMP factor, a pEcoli-6 × HN-GFPuv and pEcoli-6 × HN-CAMP factor plasmids were transformed into *E. coli*, BL21 (DE3) competent cells. A 2 ml aliquot of the overnight culture was added in 200 ml LB medium (1:100 dilution) and incubated at 37 °C until reaching optical density at 600 nm ($OD_{600}$) of 0.6. Subsequently, Isopropyl-β-D-thiogalactoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) was added into the culture to a final concentration of 1 mM for 4 h. After centrifugation at 3000 × *g* at 4 °C for 5 min, bacterial *pellets* were resuspended with 6 M urea. The supernatant was collected by centrifugation at 13,000 × *g* for 20 min then loaded onto a column with 2 ml Ni-NTA agarose (QIAGEN, Valencia, CA, USA), which had been equilibrated previously with Buffer A (20 mM Tris–HCl, 0.5 M NaCl, 1 mM 2-mercaptoethanol, pH 8.0) containing 6 M urea. The column was washed sequentially with 5 ml aliquots of Buffer A containing 6–0 M urea gradient. The bound fraction was then eluted with 5 ml of Buffer A containing 500 mM Imidazole. The purified and refolded proteins were dialyzed overnight at 4 °C in 5 l of phosphate-buffered saline (PBS) by using Spectra/Por molecular porous membrane tubing [molecular weight cut off (MWCO): 3500] (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) and then concentrated by *lyophilization*. A 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and coomassie blue staining were used to determine the protein expression.

### 2.2. CAMP factor-induced inflammation

An amount of 50 μg purified CAMP factor in 25 μl PBS was intradermally injected in the central portion of the right ear. As a control, purified GFP was injected into the left ear of the same mice. To prevent leakage, proteins were gradually injected into mouse ears using a 28-gauge needle followed by a slow withdrawal of the