Targeting a bacterial defense system: a new paradigm for antimicrobial therapy

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Public health problems caused by infectious diseases

- Infections are #3 cause of death in the US.
- Infections are #2 cause of death in the world (14.9 million in 2004, 29% of all death).
- Hospital acquired infections: 2 million per year in US (90,000 deaths)

(WHO World Health Report, CDC, 2004)
**Staphylococcus aureus**

- Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has large, round, and golden-yellow colonies.
- A spherical bacterium, frequently found in the nose and skin of a person.
- *Staphylococcus aureus* is a virulent *pathogen* that is currently the most common cause of infections in hospitalized patients and also community-acquired infections.

[Gram stain of *Staphylococcus aureus* in pustular exudate](http://www.textbookofbacteriology.net/staph.html)

[Microscope picture of *Staphylococcus aureus*](http://www.kimicontrol.com/edu-e.html)
Bacterial infections

- Nervous system
- Eyes & Ears
- Skin
- Respiratory system
- Gastrointestinal tract
- Urogenital system
- Blood & whole body
S. aureus infections

- Boils, carbuncle, furunculosis, styes: superficial skin lesion
- Cellulitis(subcutis), pneumonia(lung), mastitis(mammary gland), phlebitis(vein), meningitis(brain), urinary tract infection, osteomyelitis(bone), endocarditis(heart), septicemia (bloodstream)
- Food poisoning
- Toxic shock syndrome
- Noscomial infection (hospital acquired infection)
US Health burden of *S. aureus*

- Community-acquired infections
- Hospital-acquired infections
  (second leading cause of nosocomial bloodstream infections)
- 12,000 excess deaths per year
- 2.7 million excess hospital days
- 9.5 billion excess dollars

(Naskin et al., Arch Internal Medicine 2005; 165;1756-1761)
Development of Antibiotics

Penicillin by A. Fleming

“The mold *Penicillium* prevents the multiplication of Staphylococci” (1928)

“Miracle drug”

**Fleming’s original plate:**

- mold
- bacterial colonies

Before Penicillin treatment

After Penicillin treatment
Antidrug resistance

- Loss of effectiveness of a pharmacological agent against a pathogen.

*Antibiotic deployment*

*Antibiotic resistance observed*


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The rise of the superbug

• Methicillin-resistant \textit{S. aureus} (MRSA)
  • Producing $\beta$-lactamase
  • Containing a gene coding for an additional penicillin binding protein which has only low affinity for the $\beta$-lactom antibiotics.
  • Showing resistance to more than 40 $\beta$-lactom antibiotics.

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What are the main problems with MRSA?

- Increasing prevalence of multidrug-resistant *S. aureus*
- Increasing number of MRSA infection cases
- The need for new antibiotic is getting increased, but antibacterial drug approvals are declining.

- Dr. Anthony Fauci, director of the National Institute of Allergy and Infectious Diseases at NIH, referred to MRSA as a “global pandemic” (*48th Interscience Conference on Antimicrobial Agents and Chemistry*, 2008).
Global pandemic of MRSA infection (2003)
Invasive MRSA infection in the US was 31.3 per 100,000 (2005)
94,360 invasive MRSA cases in the US (2005)
- 18,650 of them fatal (vs. 17,000 for HIV disease)

(Klevens et al., JAMA 2007, 298;1763-1771)
FDA approvals of new antibiotics are declining

(Spellberg et al., CID 2004, 38;1279-1286 (modified))
When a superbug strikes close to you, How will you deal with it?

Case 1: Vance McGaugh was a normal healthy teenager. As his parents stood by his side at Cook Children's Medical Center, they kept expecting him to pull through. But that didn't happen. By the time he made it to the hospital it was too late. Now his parents hope his story will save others from the "superbug" MRSA. (Huffington Post, April 3, 2008)

Case 2: Michael Jackson has contracted an MRSA-type skin infection during plastic surgery to reconstruct his nose, it has emerged. There’s a chance it could turn into a flesh-eating disorder where it begins to kill-off his skin so he’s being very carefully monitored. (Mail online, Feb 12, 2009; www.dailymail.com)
- Increasing prevalence of multidrug-resistant *S. aureus*
- Increasing number of MRSA infection cases
- The need for new antibiotic is getting increased, but antibacterial drug approvals are declining.
A new strategy for antimicrobial therapy

- **DISARMING** instead of **KILLING** PATHOGENS

- Anti-virulence or anti-defense therapies:
  - Inhibiting virulence factors or bacterial defense systems
  - Similar strategy to vaccine therapy for virulence factors
  - The bacteria will be cleared by the host immune response.

- **Advantage**
  - New classes of antibiotics: resistance-free
  - Expanding the repertoire of bacterial targets
  - Imposing weaker selective pressure for the development of resistance
  - Preserving the host endogenous microbiome

- **Disadvantage**
  - They might need to be combined with antibacterial agents to achieve their full potential.
Phagocytosis and bacterial defense

- Oxygen-dependent degradation in phagolysosome depends on NADPH and the production of reactive oxygen species.
- Hydrogen peroxide and myeloperoxidase activate a halogenating system which leads to the destruction of bacteria.

ROS defense system in *S. aureus*
Can ROS defense system of *S. aureus* be a target for antibacterial therapy?
A cholesterol biosynthesis inhibitor blocks *S. aureus* virulence

- SQS inhibitors blocked staphyloxanthin biosynthesis *in vitro*.
- Staphyloxanthin has a key role in ROS defense.
- Inhibitors result in colorless bacteria with increased susceptibility to killing by human blood and to innate immune clearance in a mouse infection model.


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Antibiotics with diverse targets (ribosome for aminoglycosides, DNA gyrase for quinolone and penicillin-binding proteins for β-lactam) trigger NADH depletion and superoxide (O$_2^-$) formation by hyperactivation of the electron transport chain. Free-radical damage of iron-sulfur clusters releases ferrous ion, inducing the generation of highly destructive hydroxyl radicals 1(OH) and cell. 

*Molecular Systems Biology* **3**: 142, 16 October 2007
Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy

**Figure A**
- Intraperitoneal *E. coli*
- No treatment
- No phage + oflox
- *ϕ* + oflox
- *ϕ* + oflox

**Figure B**
- Mean killing (Δlog CFU/mL)
- Treatment time (h)

**Figure C**
- Survival percentage
- Treatment time (d)

*Timothy et al., (2009) PNAS. 106(12) 4629*

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Combination drugs, an emerging option for antibacterial therapy

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Summary

- Targeting against the bacterial defense systems or virulence systems is the alternative way to develop the new-generation antibiotics that can be used directly or in combined with known antibiotics.
Structure-based identification of a novel histidine kinase that has a role in ROS defense
Quorum sensing system in *S. aureus*

- TRAP is a key player in quorum sensing signaling and virulence.

**Autoinducer:** RAP, AIP
**AgrA:** TRAP dephosphorylation
TRAP activates the RNAII transcription

**Quorum system**

- **agrA**
- **agrC**
- **agrD**
- **agrB**

**Toxin production**

- RNAII
- RNAIII

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TRAP was considered as a new target for developing antibiotics.

   Treatment of Staphylococcus aureus Biofilm Infection by the Quorum-Sensing Inhibitor RIP.
   PMID: 17371825 [PubMed - in process]

2. Korem M, Gov Y, Kiran MD, Balaban N.
   Transcriptional profiling of target of RNAIII-activating protein, a master regulator of staphylococcal virulence.
   PMID: 16177293 [PubMed - indexed for MEDLINE]

   Quorum sensing in Staphylococci is regulated via phosphorylation of three conserved histidine residues.
   PMID: 14726534 [PubMed - indexed for MEDLINE]

   Characterization of RAP, a quorum sensing activator of Staphylococcus aureus.
   PMID: 12829282 [PubMed - indexed for MEDLINE]

   Use of the quorum-sensing inhibitor RNAIII-inhibiting peptide to prevent biofilm formation in vivo by drug-resistant Staphylococcus epidermidis.
   PMID: 12599079 [PubMed - indexed for MEDLINE]

   Regulation of Staphylococcus aureus pathogenesis via target of RNAIII-activating Protein (TRAP).
   PMID: 11160124 [PubMed - indexed for MEDLINE]
TRAP is a master regulator of staphylococcal virulence

<table>
<thead>
<tr>
<th>Virulence determinant</th>
<th>Gene</th>
<th>Influence of:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>trap</td>
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<tr>
<td>Exotoxins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aureolysin</td>
<td>awr</td>
<td>+</td>
</tr>
<tr>
<td>Capsular polysaccharide type 5 genes</td>
<td>cap</td>
<td>+</td>
</tr>
<tr>
<td>V8 protease</td>
<td>spaA</td>
<td>+</td>
</tr>
<tr>
<td>Toxic shock syndrome toxin</td>
<td>tcp</td>
<td>unknown</td>
</tr>
<tr>
<td>Glycerol ester hydrolase</td>
<td>geh</td>
<td>+</td>
</tr>
<tr>
<td>α-Hemolysin</td>
<td>kia</td>
<td>+</td>
</tr>
<tr>
<td>β-Hemolysin</td>
<td>kib</td>
<td>+</td>
</tr>
<tr>
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<td>kigB</td>
<td>+</td>
</tr>
<tr>
<td>8-Hemolysin</td>
<td>kld</td>
<td>+</td>
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<tr>
<td>Hyaluronate lyase</td>
<td>hydA</td>
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<tr>
<td>Lipase</td>
<td>hp</td>
<td>+</td>
</tr>
<tr>
<td>Holin-like proteins</td>
<td>hbg</td>
<td>+</td>
</tr>
<tr>
<td>1-Phosphatidylinositol phosphodiesterase</td>
<td>nic</td>
<td>+</td>
</tr>
<tr>
<td>Surface-adhesion molecules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein A.d.a.</td>
<td>spa</td>
<td>−</td>
</tr>
<tr>
<td>Coagulate</td>
<td>coa</td>
<td>Ø</td>
</tr>
<tr>
<td>Fibrinogen-binding protein</td>
<td>sdrC</td>
<td>Microarray</td>
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<td>Real time</td>
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<td>Fibrinogen-binding protein</td>
<td>sdrD</td>
<td>Microarray</td>
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<tr>
<td></td>
<td></td>
<td>Real time</td>
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TRAP is a key player in *S. aureus* pathogenicity

- Mouse skin infection (cellulitis) model. TRAP(-) or His-mutants showed no lesion. *J Biol Chem.* 2004 Apr 9;279(15):14665-72.

- Biofilm formation can be suppressed in TRAP(-).

**TRAP is a useful vaccine target**

- Antibodies to TRAP have been shown to suppress exotoxin production by *S. aureus in vitro*, suggesting that TRAP may be a useful vaccine target site.

- Mice immunized with TA21 were protected from *S. aureus* infection

- The level of hot-resistant exotoxins was decreased by antibodies against FTA21

But, TRAP has no role in *S. aureus* quorum sensing!!

- **Mutation of *traP* in *Staphylococcus aureus* has no impact on expression of *agr* or biofilm formation.**

- **Inactivation of *traP* has no effect on the *agr* quorum-sensing system or virulence of *Staphylococcus aureus*.**

• **Then, what is the role of TRAP**
Our approach: structural proteomics

Molecular Function: Phosphorylation of the substrate protein

Cellular Function: Signal transduction in immune response

Jak3 kinase
Structure determination using X-ray crystallography

1. 대상유전자 클로닝
2. 대장균을 이용한 단백질발현 및 정제
3. 결정성장

6. 삼차원구조완성(구조-기능연구)
5. 컴퓨터를 이용한 전자밀도해석
4. X선을 이용한 회절데이터수집
Crystal structure of TRAP from *S. aureus*

- Crystal structure of TRAP at 1.8 Å resolution.
From the crystal structure of TRAP, we have identified that TRAP is a novel histidine kinase with DNA protection activity against ROS, and thus designated it as DPK (DNA Protection Kinase).
Function prediction from the 3D structure

- The C-terminal domain of DPK
- NtrB; nitrogen sensing histidine kinase of *E.coli*
- The N-terminal domain of DPK
- E2 viral DNA binding domain

Is it a novel protein kinase?

Is it a novel DNA binding protein?
Yes, DPK is a novel His protein kinase

- Its function is activated by Fe$^{2+}$ and hydroxyl radicals.
Both hydroxy radicals and irons are required for full DPK activity.

<table>
<thead>
<tr>
<th>+ hydroxyl radical</th>
<th>- hydroxyl radical</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Mg²⁺</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>Fe²⁺</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>Fe³⁺</td>
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1 mM 2,2-Dipyridyl
Yes, DPK is a DNA binding protein

- Its function is activated by Zn$^{2+}$
What is the role of DPK?

- The role of DPK might be to protect DNA against oxidative stress or DNase.
How does DPK protect DNA?

- DPK might protect DNA by compacting DNA conformation or by histone-like function.
What is the biological meaning of DPK phosphorylation

- The DNA protection activity of DPK is enhanced by phosphorylation.
DPK has a role in oxidative-stress defense in bacteria

![Graphs showing the effect of H$_2$O$_2$ on bacterial survival in wild and Δdpk strains.](image)

**Left panel:**
- **Y-axis:** Log$_{10}$ cfu/ml
- **X-axis:** H$_2$O$_2$ (mM)
- (Wild and Δdpk lines showing different survival rates)

**Right panel:**
- **Y-axis:** Log$_{10}$ cfu/ml
- **X-axis:** time (hr)
- (Wild and Δdpk lines showing different survival rates under 2 mM H$_2$O$_2$)

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The role of DPK was confirmed in DPK-overexpressed *E. coli* cells.
Antibiotics with diverse targets (ribosome for aminoglycosides, DNA gyrase for quinolone and penicillin-binding proteins for β-lactam) trigger NADH depletion and superoxide (O$_2^-$) formation by hyperactivation of the electron transport chain. Free-radical damage of iron-sulfur clusters releases ferrous ion, inducing the generation of highly destructive hydroxyl radicals (OH) and cell.

*Molecular Systems Biology* 3: 142, 16 October 2007
DPK has a role in antibiotics defense in bacteria

![Graph showing bacterial growth](image)

- **Wild** and **Δdpk** strains of bacteria were exposed to different concentrations of Norfloxacin. The graphs illustrate the log$_{10}$ of cfu/ml over time (hr) at a fixed concentration of 5 μg/ml Norfloxacin.

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Can **DPK** be a target for antibacterial therapy?
**DPK mutant is sensitive to antibiotics in thigh infection model**

<table>
<thead>
<tr>
<th></th>
<th>Initial Con.</th>
<th>0mpk</th>
<th>20mpk</th>
<th>40mpk</th>
<th>80mpk</th>
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</thead>
<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td>5.94 ± 0.3</td>
<td>9.11</td>
<td>9.11</td>
<td>8.51</td>
<td>8.28</td>
</tr>
<tr>
<td><strong>dpk mutant</strong></td>
<td>5.82 ± 0.3</td>
<td>8.49</td>
<td>8.23</td>
<td>7.62</td>
<td>6.87</td>
</tr>
</tbody>
</table>

![Graph showing the comparison of log CFU/thigh between Wild type and dpk mutant after different doses of Norfloxacin.](attachment:image.png)
DPK mutant is sensitive to antibiotics in mouse survival test

1. Infection (abdominal cavity)  
2. Antibiotics (oral)  
3. Survival test (days 1, 2...)

Survival rates of mice infected with wild type S. aureus

Survival rates of mice infected with Δdpk S. aureus

Time after infection (days)

Survival rate (%)

Norfloxacins 80mg/kg, 40mg/kg, 20mg/kg, No treatment
Role of TRAP (DPK) in ROS defense

Role of TRAP in quorum signaling

Autoinducer: RAP, AIP
AgrA: TRAP dephosphorylation
TRAP activates the RNAII transcription

Toxin production
DPK inhibitors
In vitro phosphorylation assay (KRICT chemical library)

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Dimerization of DPK might be necessary for its activation

- C-terminal residues seem to be important for dimerization.

- DPK forms a dimer

- Dimer model of DPK

- C-terminal Peptide
Peptide based virtual screening, a peptide mimetics

Reference peptide

Virtual screening

10 candidate chemicals
In vitro phosphorylation test using the DPK inhibitor candidates

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<tr>
<th></th>
<th>Con</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>5</th>
<th>6</th>
<th>7 (candidate chemicals)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100 μM</td>
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</tr>
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DPK inhibitors increase antibiotic susceptibility of MRSA
Summary

- The crystal structure of DPK was determined at 1.8 Å resolution
- The biochemical function of DPK was successfully inferred from the structural information.
- DPK is a novel histidine kinase with DNA protection activity against ROS.
- ROS induced the histidine phosphorylation of DPK, which in turn enhanced its DNA protection activity.
- DPK(-) cells showed increased sensitivity to antibiotics and hydrogen peroxide.

DPK plays a role in ROS and antibiotics defense by protecting bacterial genome from ROS, and thus it is confirmed to be a target for antibacterial therapy.

- DPK inhibitors were developed by virtual and chemical screening using phosphorylation assay.
- DPK inhibitors elevated the sensitivity of *S. aureus* to antibiotics.
- DPK inhibitors can be developed as a new-type of antibiotics or an adjuvant.

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