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Review

Targeting ADP-ribosylation as an antimicrobial strategy

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ABSTRACT

ADP-ribosylation (ADPr) is an ancient reversible modification of cellular macromolecules controlling major biological processes as diverse as DNA damage repair, transcriptional regulation, intracellular transport, immune and stress responses, cell survival and proliferation. Furthermore, enzymatic reactions of ADPr are central in the pathogenesis of many human diseases, including infectious conditions. By providing a review of ADPr signalling in bacterial systems, we highlight the relevance of this chemical modification in the pathogenesis of human diseases depending on host-pathogen interactions. The post-antibiotic era has raised the need to find alternative approaches to antibiotic administration, as major pathogens becoming resistant to antibiotics. An in-depth understanding of ADPr reactions provides the rationale for designing novel antimicrobial strategies for treatment of infectious diseases. In addition, the understanding of mechanisms of ADPr by bacterial virulence factors offers important hints to improve our knowledge on cellular processes regulated by eukaryotic homologous enzymes, which are often involved in the pathogenesis of human diseases.

1. Introduction

ADP-ribosylation (ADPr) is a reversible regulatory mechanism widespread in viruses, bacteria and in the large part of eukaryotes [1–5]. ADPr is catalysed by enzymes transferring ADP-ribose unit(s) from nicotinamide adenine dinucleotide (NAD^+) mainly onto cellular protein substrates with the release of nicotinamide [5]. However, additional cellular macromolecules, such as DNA and antibiotics, are target of ADPr [6–8]. Throughout the evolution, ADPr has evolved in order to modulate a multitude of vital physiological processes, such as DNA damage repair [9–11], transcription [12–15], cell division [16–21], proliferation [22–25] and cell death [26–30], to cite a few. Additionally, ADPr is involved in the pathogenesis of many human diseases as well, which include neurological disorders, cancer, and bacterial- and viral- mediated infections [4,31–36].

In the context of translational research, cellular mechanisms regulated by ADPr have attracted the attention of the scientific community, especially in the field of cancer research where the modulation of endogenous systems of ADPr has been recognised effective in sensitising cancer cells to death [37–39]. Furthermore, because of the medical need, the understanding of virulence mechanisms exploiting ADPr systems is considered equally of interest.

The cellular enzymes catalysing ADPr are named ADP-ribosyl transferases (ARTs) [40–44]. Diphtheria (DTX) and Cholera (CTX) exotoxins, respectively produced by pathogenic *Corynebacterium diphtheriae* and *Vibrio cholerae*, represent the ancestors of ART superfamily of enzymes [42,45], as well as the prototype of toxic ART exoenzymes [31,46–55]. Bacterial ARTs (bARTs) are well known causative agents of

Abbreviations: ADPr, ADP-ribosylation; NAD^+, nicotinamide adenine dinucleotide; ARTs, ADP-ribosyl transferases; DTX, diphtheria toxin; CTX, cholera toxin; bARTs, bacterial ARTs; PAR, poly(ADP-ribose); SIRTs, sirtuins; MARylation, mono(ADP-ribosyl)ation; PARylation, poly(ADP-ribosyl)ation; ARTDs, ADP-ribosyl transferases diphtheria toxin-like; ARTCs, ADP-ribosyl transferases cholera toxin-like; PARPs, poly(ADP-ribose) polymerases; ER, endoplasmic reticulum; ARTT, ADP-ribosylating turn-turn; A-loop, acceptor-loop; D-loop, donor-loop; MTX, mosquitocidal toxin; DarTG, DNA ART/DNA ADP-ribosyl glycohydrolase; TA, toxin-antitoxin; PTMs, post-translational modifications; HPF1, histone PARylation factor 1; ARHs, ADP-ribosyl-acceptor hydrolases; DraG, dinitrogenase reductase-activating glycohydrolase; PARG, poly(ADP-ribosyl) glycohydrolase; TARG1, Terminal ADP-ribose glycosylhydrolase 1; CoVs, coronaviruses; LT, heat-labile enterotoxin; PT, pertussis toxin; GPCRs, G-protein-coupled receptors; cAMP, cyclic-AMP; PKA, protein kinase A; CFTR, cystic fibrosis transmembrane conductance regulator; CDT, C. difficile toxin; CST, C. spiroforme toxin; ExoS, exoenzyme S; ExoT, exoenzyme T; RhoGAP, Rho GTPase activating protein; ERM, ezrin, radixin, and moesin proteins; PKC, protein kinase C; EF2, elongation factor-2; ChT, Cholix toxin; PDE, phosphodiesterase; Nudix, nucleoside diphosphate-linked moiety X; NPP, nucleotide pyrophosphatase/phosphodiesterases; RTN4, reticulon-4; Prs, phosphoribosyl pyrophosphate synthetase; Tre1, type VI secretion ADP-ribosyl transferase effector 1; BFA, brefeldin A; BARS, BFA-ADP-ribosylation substrate; CtBP1-S, C-terminal-binding protein-1 short-form; BAC, BFA-ADP-ribose conjugate; ARF, ADP-ribosylation factor; GEFs, guanine nucleotide exchange factors; GDP, guanosine diphosphate

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large number of worldwide spread diseases, affecting humans, insects and plants [31,56–58], with a great impact on human health. In addition, infectious diseases strongly affect the world economy in terms of costs for the human health as well as food and agricultural economic losses [59]. The use of antibiotics to counteract the pathogen infections has represented the therapeutic strategy of choice in the last century, however the emergence of antibiotic resistance strains represents the major challenge of the 21st century [60–62]. Targeting bacterial pathogenic mechanisms by disarming pathogens of virulence factors, for instance by inhibiting the enzymatic activity of bART, represents a valid alternative intervention strategy to overcome antibiotic resistance [63–66]. In addition, because of the conservation of ADPr systems throughout the evolution, the understanding of bacterial pathogenic mechanisms may contribute to unveil novel and conserved cellular processes regulated by ADPr in high organisms [34,45,67,68]. The dysregulation of such processes can be either cause of human diseases or be target of therapeutic intervention [39,69–71].

Herein, we will provide a summary of bacterial ADPr systems describing their pathogenic mechanisms and highlighting the similarities with ADPr systems in mammals as well. Further, we discuss the potential of targeting ADPr for therapeutic strategies of infection diseases.

2. ADP-ribosylation in pathophysiology

ADPr was originally described in the 60s; two independent studies reported the identification of a new polymer, poly(ADP-ribose) (PAR) synthesised from NAD^+ in vertebrate cells [72] and, simultaneously, the finding that bacterial DTX activity from *C. diphteriae* is dependent on NAD^+ content [73]. In the following decades, research in the field has expanded the involvement of ADPr in the pathophysiology of diverse cellular processes in all domains of life, which include DNA damage repair [9–11,14,74–78], cell metabolism [78–80], aging [81], cell division [16–21], transcription [12–15], cell death [26–30], bacterial and viral pathogenic mechanisms [31,33,35], defence against infection [4,24,32–34,82–84], stress response [85–89], and many others emerging pathways as intracellular trafficking [90] to refer to human cellular functions.

Because of the central role of ADPr in many essential cellular processes, the cellular signalling controlled by ADPr is finely tuned by the activity of ADP-ribosyl hydrolases. Thus, ADPr is a reversible modification. Dysregulation of ADPr signalling as well as the unbalance between transferases' and hydrolases' activities has proven to have a role in many inherited and acquired human diseases, as in several neurological disorders and in cancer [36,39,70,91–95].

3. Enzymes involved in ADPr signalling

3.1. Transferases

Two evolutionary unrelated superfamilies of enzymes catalyse ADPr; ARTs [42,45] and Sirtuins (SIRTs) [96]. In this review we will not discuss about the SIRT enzymes. The majority of proteins belonging to the ART and SIRT superfamily of enzymes covalently transfer single ADP-ribose units to target proteins, thus producing mono(ADP-ribosyl)ation (MARylation) reaction [43,97]. In addition, several ARTs can transfer chains of repeating ADP-ribose units (up to 200 in length) giving rise to PAR polymers, as a result of poly(ADP-ribosyl)ation reaction (PARylation) [9,43,45,98].

3.1.1. ADP-ribosyl transferases (ARTs)

ART enzymes are widely distributed across all domains of life from bacteria to humans with exception of yeasts [2,5,31,97] and, according to the structural organisation of the ART fold, are subdivided into diphtheria toxin-like (ARTDs) and cholera toxin-like ARTs (ARTCs) classes [42,45]. Despite low sequence similarity, the two classes of ART domains share a common conserved secondary structure and protein fold [3,42,45,54]. Diverging from the NAD^+-binding Rossmann fold, which features oxidoreductase enzymatic activities [3], the ART protein fold consists of two central β-sheets surrounded by α-helices, with the NAD^+ binding pocket located at the interface of the two slightly staggered β-sheets [51,99]. Three amino acids within the ART fold form a triad essential for enzymatic catalysis. The H-Y-E triad is a feature of the ARTD family, whilst the R-S-E residues characterise the ARTC group of enzymes. In detail, the histidine in position one of the ARTD's catalytic triad (H-Y-E) binds to the 2-OH of the adenosine ribose and the NH_2 of the nicotinamide amide, the tyrosine in position two π-stacks with the nicotinamide ring, and the glutamate in position three is supposed to stabilise the furanosyl oxocarbenium intermediate. Mutation of the glutamate residue in the active site of DTX decreases catalytic activity leading to loss of cytotoxicity [100–102]. In eukaryotes, ARTD enzymes are extensively known as Poly(ADP-ribose) polymerases (PARPs). The human genome encodes seventeen PARPs, which, based on variation in their catalytic triad, are divided in five groups. The first group encompasses the H-Y-E-containing enzymes (PARP1, PARP2, PARP3, PARP4, PARP5a and PARP5b). PARP1 and PARP2 catalyse synthesis of linear polymers of PAR (up to 200 units in length) through the formation of glycosidic ribose-ribose 1"-2" bonds [43,103], or of branched portions of PAR by the formation of glycosidic ribose-ribose 1″-2″ linkages [104–106]. PARP5a and PARP5b (Tankyrase-1 and -2, respectively) catalyse the formation of PAR oligomers by addition of repeating units of ADP-ribose (up to 20 units in length) [9,43]. Instead, PARP3 and PARP4 catalyse MARylation [43]. Additional groups of human PARPs are the H-Y-I triad-containing enzymes (PARP6, PARP7, PARP8, PARP10, PARP11, and PARP12), the H-Y-Y-containing PARP16, the H-Y-L-containing PARP14 and PARP15, and the Q-Y-T/Y-Y-T-containing PARP9 and PARP13. With the exception of PARP13, which appears to be inactive [42,43,107], and of true poly(ADP-ribose) polymerases, the remaining human ARTD/PARP enzymes catalyse MARylation of their targets [43,108]. In addition, a divergent PARP-like enzyme containing the triad H-H-V belongs to a subgroup within the eukaryotic ARTD class [3,42] and it is referred as TpT1 or KptA (ARTD18).

ARTCs are characterised by the R-S-E triad in the catalytic domain. Similar to the histidine within the ARTD triad, the arginine in the position one of the R-S-E triad interacts with the diphosphate backbone of NAD^+, the serine binds to the nicotinamide ribose, and the glutamate acts as in the H-Y-E triad. ARTCs are known for their ability to modify protein substrates on arginine residues, through N-glycoside bonds (please refer to Section 3.1.3. for further details). Four ecto-ARTs (hARTC1, hARTC3, hARTC4, hARTC5) are expressed in humans, and six in mice (Artc1, Artc2.1, Artc2.2, Artc3, Artc4, and Artc5) [78,97,109,110]. Ecto-ARTs are glycosylphosphatidylinositol (GPI)-anchored proteins, with exception of Artc5 that is a secreted enzyme. Murine Artc1, Artc2 and Artc5 selectively MARylate plasma membrane-associated proteins, such as P2X7 receptor, which once modified leads to apoptotic cell death [111,112], and extracellular proteins such as the heme-binding protein hemopexin [113–116], whose ADPr reduces its capacity to bind heme. Additionally, human ARTC1 localises at the Endoplasmic Reticulum (ER) and, by modifying GRP78/BiP, functions in ER stress response [88]. Conversely, ARTC3 and ARTC4 lack the conserved residues in the catalytic triad and hence are thought to be inactive [110]. Despite the membrane-associated localisation of ecto-ARTs, several studies revealed the ADPr of arginine (Arg-ADPr) residues of soluble intracellular proteins (*e.g.* GAPDH, G protein βγ subunits, Rabs, and tubulin) [116–119], thus suggesting that mammalian ARTCs also regulate intracellular functions.

In addition to catalytic triads, further conserved motifs are functional to the coordination of NAD^+ or to substrate selectivity. For instance, the ARTT loop (ADP-ribosylating turn-turn), also known as the acceptor-loop (A-loop), is found in both ARTD and ARTC protein families. Studies have demonstrated the central role of the A-loop in bacterial ARTCs for targeting and selection of amino acid acceptors. The donor-loop (D-loop) is instead a common feature of ARTD enzymes

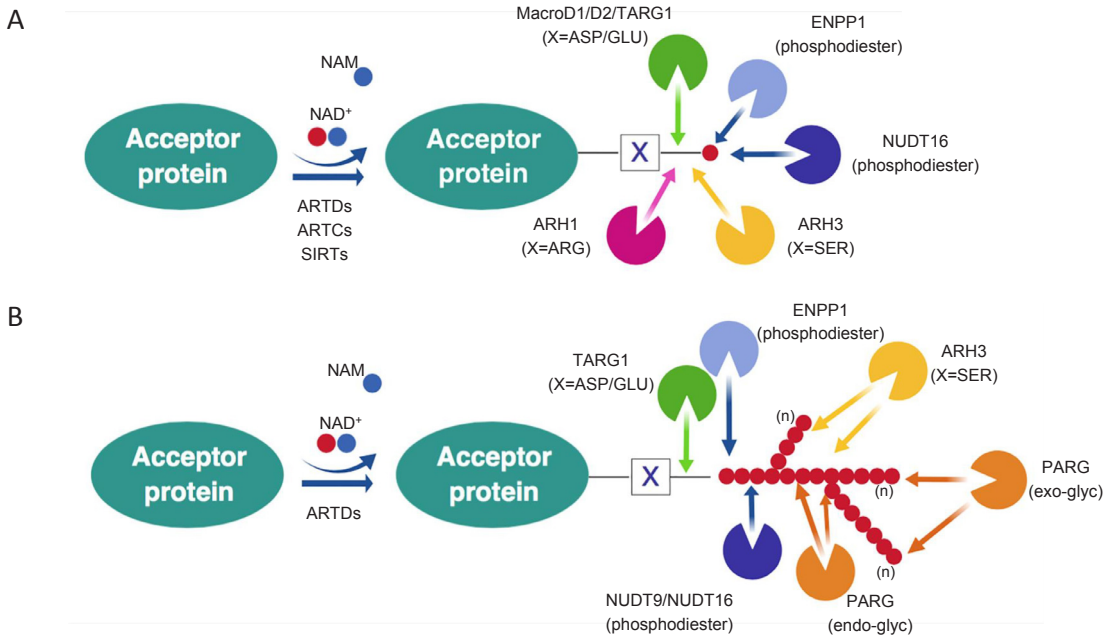

Fig. 1. Schematic representation of the ADP-ribosylation reaction onto target protein. A) Mono-ADP-ribosylation reaction is catalysed by ADP-ribosyl transferases Diphteria toxin-like (ARTDs), ADP-ribosyl transferases Cholera toxin-like (ARTCs) and Sirtuins (SIRTs) in the presence of the NAD^+. The modification, indicated as a single red ball, is reversed by selective ADP-ribose hydrolytic enzymes (MacroD1, MacroD2, TARG1, ARH1, ARH3) that show different amino acid-ADP-ribose linkage specificity, and by phosphodiesterases (NUDT16 and ENPP1). B) Poly(ADP-ribosyl)ation reaction is catalysed by ADP-ribosyl transferases Diphteria toxin-like (ARTDs). Linear or branched chains of Poly(ADP-ribose), indicated as red balls, are hydrolysed by selective ADP-ribose hydrolytic enzymes (PARG, ARH3, TARG1) and by phosphodiesterases (NUDT16 and ENPP1). Further details are reported in the text. NAM, nicotinamide; ARH1/ARH3, ADP-ribosyl acceptor hydrolases 1/3; NUDT16, nudix hydroxylase 16; PARG, Poly(ADP-ribosyl) glycohydrolase (*endo*-glyc, *endo*-glycolytic activity; *exo*-glyc, *exo*-glycolytic activity); TARG1, Terminal ADP-ribose glycosylhydrolase 1.

that is involved in NAD^+-binding and catalysis; conversely, it is not present in bacterial ARTCs and eukaryotic counterparts [42,45].

3.1.2. Substrates of ADPr

ARTs are mainly known in modifying proteins, however, several enzymes can modify additional molecules, such as nucleic acids (both DNA and RNA) and antibiotics [5–7,34,78]. The structure of bARTs is overall conserved in eukaryotic homologues belonging to same families, however, the selectivity for targets is clearly divergent throughout the evolution. Indeed, bARTs show a narrow target selection towards well defined protein or nucleic acid substrates. By contrast, eukaryotic ARTs show a broad ability in modifying different molecules as well as multiple residues within proteins substrates, as shown in Fig. 1 and further discussed in Section 3.1.3.

ADPr of DNA has been described for both bARTs and mammalian ARTs. Modification of DNA by ART toxins is performed by Pierisin-1 from cabbage butterfly *Pierisin rapae* [120], mosquitocidal toxin (MTX) from *Bacillus sphaericus* [121], ScARP from *Streptomyces coelicolor* [122], Scabin from the pathogen *Streptomyces scabies* [123], and the non-toxin enzyme Carp-1 from shellfish *Meretrix lamarckii* [124]. Pierisin-like enzymes belong to the ARTC group and irreversibly target the N2 amino groups of 2′-deoxyguanosine in double stranded DNA *in vitro*. The cytotoxic effect of Pierisin-like enzymes has been assessed in several human cell lines, where they induce an apoptotic response [125]. Therefore, Pierisin enzymes have been suggested to function in defence mechanisms. An additional example of bART modifying DNA is provided by the reversible DarTG (DNA ART/DNA ADP-ribosyl glycohydrolase) toxin-antitoxin (TA) system encoded by *Mycobacterium tuberculosis*, *Escherichia coli* and *Thermus aquaticus* [8]. The DarT toxin, which shares homology with ARTDs, modifies endogenous DNA onto thymidine residue within a conserved single stranded nucleotide sequence. As a result of the DNA replication blockade, DarT induces a reversible bacteriostatic persistence state [8]. Further details about DarTG system are provided in Section 4.2. Mammalian DNA-damage related PARPs are able to modify DNA as well. PARP1 and PARP2 transfer chains of ADP-ribose on phosphorylated DNA termini *in vitro* with differences; PARP1 PARylates ends of recessed DNA duplexes containing 3′-cordycepin, 5′- and 3′-phosphate and to 5′-phosphate of a single-stranded oligonucleotide; whilst PARP2 preferentially modifies nicked/gapped DNA duplexes containing 5′-phosphate at the double-stranded termini [126]. PARP3 has been shown to MARylate phosphorylated DNA termini *in vitro* with clear preference for 5′ ends of DNA double stranded breaks [7,127,128]. The biological significance of PARP-dependent ADPr of DNA in the context of DNA damage repair remains still unknown. However, reversible ADPr of DNA across various kingdoms of life implies that this modification may be more widespread than originally thought and could be responsible for modulating undiscovered cellular events, such as gene transcription and DNA replication.

Modification of RNAs by ADPr has been described for the divergent TpT1. *Saccharomyces cerevisiae* TpT1 is involved in RNA splicing by acting as tRNA 2′-phosphotransferase, catalysing the transfer of the 2′-phosphate from ligated tRNA to NAD^+, producing mature tRNA and ADP ribose-1″-2″-cyclic phosphate at the splice junction of tRNAs [5,129–131]. Similarly, the bacterial homologue KptA, performs same reaction *in vitro* [132], though its physiological function in *E. coli* remains largely unknown since splicing is absent. Reversible ADPr of RNAs also occurs *in vitro* by the activity of the human PARP10, PARP11 and PARP15 as well as of the human homologue of TpT1/KptA TRPT1 [133]. However, the biological relevance related to PARP-mediated RNA ADPr signalling remains yet to be unveiled.

ADPr of antibiotics has been only described for Arr-ms encoded by *M. smegmatis*, which inactivates rifamycin and derivatives by modifying the hydroxyl group at C23 of the antibiotic [134,135]. According to the structural feature of the NAD^+-binding domain, Arr-ms is a bART harbouring a divergent catalytic triad containing H-Y-D respect to the conserved H-Y-E [6]. Despite *in silico* analysis has shown that the Arr family of proteins is widely distributed in microbial communities, their

role in resistance mechanism towards antibiotics remains largely unknown.

3.1.3. Amino acids modified by ADPr

Bacterial as well as eukaryotic ARTCs are well known to MARylate protein substrates onto basic amino acid residue arginine through N-glycosidic bonds, however other residues have been identified as targets of bARTC, such as cysteine, threonine, asparagine and glutamate [136–142]. Instead, bARTDs selectively MARylate post-translationally modified histidine residues, termed diphtamide, in protein substrates (please refer to 4.1.2 for further details). Conversely, eukaryotic ARTD/PARP group modifies a multitude of proteins targeting several amino acid residues through a selective mechanism that is still unknown. Amino acids modified by eukaryotic ARTD include serine (abbreviated as Ser-ADPr) and tyrosine (Tyr-ADPr) through O-glycosylation bonds [115,143–147], negatively charged residues such as glutamic and aspartic amino acids through ester linkages [148,149], positively charged lysine through N-glycosidic bond [150], glycine as in the case of PARP9 [108] and cysteine as reported for PARP8 [43].

More than 11,000 ADP-ribosylated peptides have been mapped in mammalian (HeLa) cells in response to DNA damage by mass spectrometric approaches [151]. Of these peptides, the fraction of Ser-ADPr accounts for 90% of the total. The 30% out of 6247 Ser-ADPr peptides harbours the lysine followed by serine motif of ADPr (KS motif) [151,152]. Strikingly, modified residues within protein substrates appear spatially restricted within cellular sub-compartments, for instance Tyr-ADPr enriches at ribosomal proteins with the most significant motif consisting of a lysine residue at the +1 position of the modified tyrosine; Arg-ADPr enriches at the ER primarily onto RNA binding proteins, with the motif flanking the arginine residue enriched with serine, and histidine-ADPr enriches at the mitochondrion [151]. Importantly, residues modified by ADPr are also target of additional post-translational modifications (PTMs), such as of phosphorylation, thus suggesting an essential role for ADPr in cross-regulating PTMs in a time-dependent fashion [144,145,147,151].

While the determinant for the high selectivity of bART for substrates is not clear (further discussed in Section 4), the ability of mammalian PARPs to modify a broad (but defined) subset of substrates could be partially dependent by protein-protein interactions, which could be responsible for substrate presentation to the enzyme. Such mechanism of substrate fishing has been demonstrated for PARP5a and PARP5b [153,154] as well as for PARP6 and PARP9 [108,155]. Same as for the specificity of PARPs in targeting well defined amino acids within protein substrates. A well-studied example of specificity for modification sites within protein substrates induced by interacting proteins has been shown for PARP1. The interaction of PARP1 with histone PARylation factor 1 (HPF1) induces PARP1-dependent *in trans* modification of histones and other DNA repair factors onto serine residues, thus limiting the ability of PARP1 to automodify onto acidic residues [76,144,152].

3.2. ADP-ribosyl hydrolases

ADPr is dynamically regulated by the activity of specialised ADP-ribosyl hydrolases, which reverse the modification once the cellular response has been achieved (Fig. 1) [5,156,157]. Two evolutionary unrelated protein families are known to support this function: DraG-like ADP-ribosyl-acceptor hydrolases (ARHs) and macrodomain-containing enzymes.

DraG-like fold-containing class consists of enzymes which shares homology with the founder dinitrogenase reductase-activating glycohydrolase (DraG), firstly characterised in bacteria [158–167]. DraG is a Mg^{2+}-dependent arginine-hydrolase known to counteract the activity of the ART endotoxin DraT in the photosynthetic bacterium *Rhodospirillum rubrum* [168] (detailed in Section 4.2). The human genome encodes three DraG-related ARHs (ARH1, ARH2, and ARH3), which play roles as regulators of cellular stresses [157,161,163,164]. Similarly to bacterial DraGs, ARH1 reverses arginine ADPr operated by mammalian ARTCs as well as bacterial toxins [162,167] In this regards, ARH1-deficient mice, exhibited enhanced sensitivity to Cholera toxin [169,170], thus suggesting that modification of cellular substrates performed by bARTs can be reverted by mammalian hydrolases [162]. ARH3 shows activity in hydrolysing O-glycosidic bond, and it is highly effective in reversing Ser-ADPribosylated substrates [164], while ARH2 appears to be inactive.

Macrodomain-containing proteins share a common ADP-ribose binding domain, known as macrodomain. Macrodomains are widely distributed in all kingdoms of life from viruses to humans, suggesting their evolutionary conservation [156,171] and play crucial roles in the recognition and hydrolysis of ADPr in different cellular contexts [172]. Macrodomain-containing proteins coupled to mass spectrometry analysis have represented so far a powerful tool for *in vitro* identification of MARylated or PARylated substrates [75,118,145,151,173–175]. In addition to the ability of interacting with ADP-ribose or PAR or O-acetyl-ADP-ribose, several macrodomains have acquired ADP-ribosyl hydrolase activity, showing distinct substrate specificity. Macrodomain-containing hydrolases include the poly(ADP-ribosyl) glycohydrolase (PARG), MacroD1, MacroD2 and Terminal ADP-ribose glycosylhydrolase 1 (TARG1) [156,171,172,176–178]. PARG is the only macrodomain-containing enzyme that efficiently cleaves PAR chains through exo- or endo-glycosyl hydrolytic activity, though it is unable to remove the terminal ADP-ribose linked to protein substrates [176]. Conversely, MacroD1, MacroD2, as well as TARG1 specifically revert MARylation by hydrolysing the bond between the terminal ADP-ribose and amino acid side chain residues on acidic residues [92,156,179,180]. Macrodomain-containing hydrolases have been also found in bacterial genomes, though their functional role in bacterial metabolism is poorly investigated [181,182]. Bacterial macrodomain-containing hydrolases prevalently revert MARylation. However, some macrodomains, such as the TARG1-type macrodomain enzyme from *Fusobacterium mortiferum* ATCC 9817 [183] and the bacterial PARG from *Thermomonospora curvata* [176], have been described for the capability of hydrolysing chains of ADP-ribose, which can be produced by bacterial PARPs, such as by the *Herpetosiphon aurantiacus* PARP [176]. In addition, an endogenous bacterial PARG processing enzyme activity has been characterised in *Deinococcus radiodurans* to counteract the huge amounts of PAR accumulated after UV irradiation [184]. The identification of enzymes able to produce and degrade polymers of ADP-ribose suggests that, similarly to human counterparts, PARylation occurs in certain bacteria and it may have roles in response to genotoxic stresses.

In addition, macrodomain-containing hydrolases are encoded by viral genomes. By reversing defensive host ADPr mechanisms, viral macrodomains have been reported to support replication of viruses and their pathogenic mechanisms, thus leading to evasion of the host immune response [33,35,185]. Of note, macrodomain-containing hydrolases encoded by members of *Coronaviruses* (CoVs) have been also reported to modulate the production of interferon by erasing the ADPr-dependent signalling of the host that is required for interferon induction [186]. For further details about viral macrodomains, we refer the reader to valuable and specialised reviews in the field [33,35,185].

4. ADPr reaction in bacteria

Bacterial ARTs are involved in the regulation of bacterial metabolism and growth as well as in infection processes [56,187]. While the endotoxins act modifying bacterial effectors allowing bacteria to survive under unfavourable environmental conditions, the exotoxins are instead virulence factors that, by transferring ADP-ribose onto host targets, contribute to bacterial pathogenesis (Table 1).

Table 1
Bacterial exo- and endo-toxins with ADP-ribosyl transferase activity.

Exotoxins

Bacterium	Toxin	Target	Effect	Role in pathogenesis	Reference
CTX-like Toxin modifying G proteins					
Vibrio cholerae	CTX	Gαs (Arg201)	Gαs activation	cAMP induction	[192]
E. coli (ETEC)	ET	Gαs (Arg201)	Gαs activation	cAMP induction	[193]
Bordetella pertussis	PT	Gαi (Cys351)	Gαi inactivation	cAMP induction	[195]
CTX-like Toxins modulating actin cytoskeleton and intracellular trafficking					
C2-like					
Clostridium botulinum	C2	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[211]
Clostridium perfringens	Iota	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[212]
Clostridium difficile	CDT	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[213]
Clostridium spiroforme	CST	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[213]
Bacillus cereus	VIP	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[213]
Salmonella sp.	SpvB	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[214]
Photorhabdus luminescens	Photox	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[137]
Aeromonas hydrophila	VgrG1	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[215]
Neisseria meningitidis	Nar E	Actin	Actin Depolymerisation	Alteration of cytoskeleton	[200]
Aeromonas salmonicida	AexT	Actin (Arg177)	Actin Depolymerisation	Alteration of cytoskeleton	[216]
Photorhabdus luminescens	Tc (TccC3 Subunit)	Actin (Thr148)	Actin clustering	Alteration of cytoskeleton	[137]
C3-like					
Clostridium botulinum	C3bot	Rho A/B/C (Asn41)	Rho protein inactivation	Alteration of cytoskeleton	[210]
Staphylococcus aureus	C3Stau (EDIN)	Rho A/B/C (Asn41)	Rho protein inactivation	Alteration of cytoskeleton	[210]
Bacillus cereus	C3cer	Rho A/B/C (Asn41)	Rho protein inactivation	Alteration of cytoskeleton	[210]
Clostridium limosum	C3-lim	Rho A/B/C (Asn41)	Rho protein inactivation	Alteration of cytoskeleton	[210]
Photorhabdus luminescens	Tc (TccC5 subunit)	Rho A/B/C (Gln61 or 63)	Rho protein activation	Alteration of cytoskeleton	[137]
Paenibacillus larvae	Plx2	Rho A	Rho A protein inactivation	Reorganisation of actin cytoskeleton	[140]
Pseudomonas aeruginosa	ExoS	Rab7, Rab8, Rab11 (Arg) Rab5 (Arg 81, 91, 110, 120) Ras (Arg 41, 123) ERM proteins (Moesin - Arg 553, 560, 563)	Inhibition of Rab function Ras inactivation Rho protein inactivation	Inhibition of endocytosis Uncoupling Ras signal transduction Cytoskeleton alteration	[230] [234] [234]
Pseudomonas aeruginosa	Exo T	CRK I/II (Arg)	Uncoupling integrin signalling	Cytoskeleton alteration	[235]
Enteropathogenic E. coli (EPEC)	EspJ	Src (Glu310)	Src inactivation	Inhibition of phagocytosis	[141]
Salmonella enterica salamae	SeoC	Src (Glu310)	Src inactivation	Inhibition of phagocytosis	[142]
Salmonella. bongori	SboC	Src (Glu310)	Src inactivation	Inhibition of phagocytosis	[142]
DTX-like Toxins modulating machinery of protein synthesis					
Corynebacterium diphtheriae	DTX	EF2 (Diphthamide715)	Inhibition of EF2 function	Inhibition of translation	[53]
Pseudomonas aeruginosa	Exo A	EF2 (Diphthamide715)	Inhibition of EF2 function	Inhibition of translation	[237]
Vibrio cholerae	ChT	EF2 (Diphthamide715)	Inhibition of EF2 function	Inhibition of translation	[238]
Toxins altering the ubiquitin signalling					
Legionella pneumophila	SdeA	RTN4, Rab33b, Rab1, Rab6a and Rab30 (Ser)	Misregulation of Rab and RTN4 functions	Alteration of intracellular traffic	[244]

Endotoxins

Bacterium	Toxin	Target	Effect	Function	Reference
Rhodospirillum rubrum	DraT	Nitrogenase reductase (Arg101)	Inhibition of enzymatic activity	Deregulation of nitrogen fixation system	[168]
Azospirillum brasilense	DraT	Nitrogenase reductase (Arg101)	Inhibition of enzymatic activity	Deregulation of nitrogen fixation system	[168]
Mycobacterium tuberculosis	DarT	Single-stranded DNA (Thymidine)	Possibly altering DNA transcription/ replication	Down regulation of cell metabolism	[263]
Sphingobium sp	ParT	Phosphoribosyl pyrophosphate synthetase (Prs)	Inhibition of enzymatic activity	Altering nucleotide biosynthesis	[267]
Serratia proteamaculans	Tre1	FtsZ	Alteration of bacterial cytoskeleton	Bacteria cell death	[268]

4.1. ADPr as a mechanism of pathogenesis

Bacterial exotoxins exert their pathogenic role through MARylation of proteins within the host mainly involved in signal transduction, organisation of cellular cytoskeleton and membrane trafficking, and protein synthesis (Fig. 2) [188]. However, many other unknown cellular substrates in the host may be target of bART, thereby further mass spectrometric studies are required to expand the understanding of mechanisms of action operated by bART.

The bART family includes more than 35 toxin members that differ for several aspects, such as substrate specificity, host (plants, insects or human), mechanism of action, as well as protein domain organisation [31]. As such, in the majority of cases, exotoxins are composed by two domains referred as A:B. The A domain corresponds to catalytic ART domain, whilst the B domain corresponds to the “binding” portion that is responsible for the binding to specific cell receptors [31,188]. Domains are differently arranged in bART toxins, for instance DTX is a single A-B polypeptide chain, CTX is a A5B toxin, C2-like toxins are binary toxins, due to the presence of the A and B polypeptide chains independently expressed, C3-like toxins are single A polypeptide

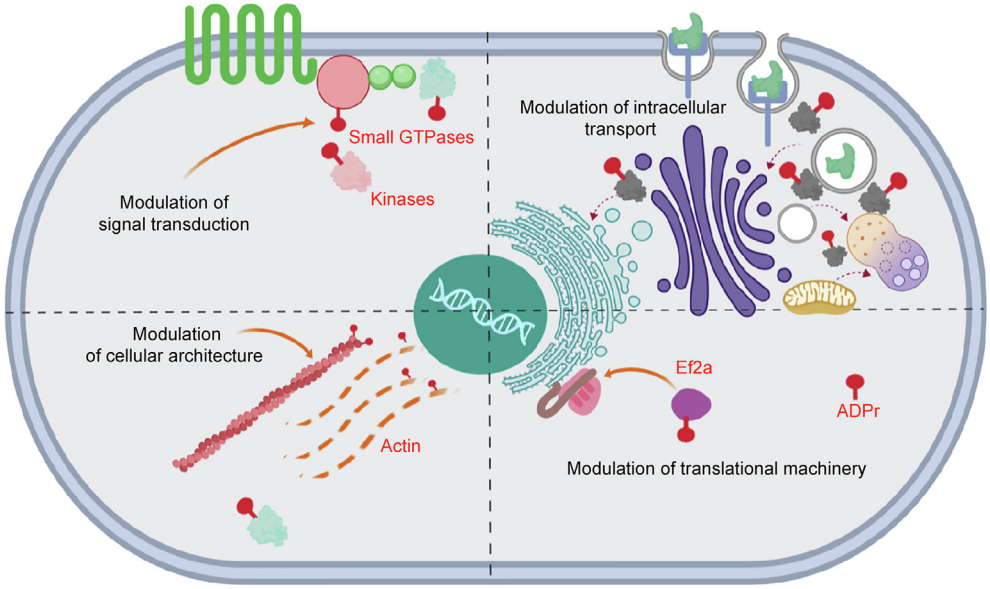

Fig. 2. Schematic representation of eukaryotic cellular mechanisms modulated by virulence factors displaying ADP-ribosylation activity.

chains, as previously reviewed [31].

Thus, despite molecular and biochemical divergences, bARTs can be roughly classified into two classes, based on sequence similarities of the catalytic ART fold domain; namely the CTX-like and DTX-like [31], as described in section 3.1.1. Accordingly, we discuss the bARTs grouping them into the two main classes and then describing the substrate specificity and the pathogenic mechanisms of representative toxins (summarised in Table1). In addition, we report with details divergent and remarkable mechanisms of non-canonical ADPr of some toxins.

4.1.1. Cholera Toxin-like

bARTCs are currently subdivided in three sub-groups reflecting the specificity of toxins for intracellular targets, namely CTX-like toxins that modify small GTPases involved in signal transduction, C2-like toxins that modify actin, and C3-like toxins that interfere with membrane trafficking of the host. In addition, other non-canonical bARTC have been identified for their ability to modify cellular kinases.

The CTX-like group of toxins includes the well-known CTX from *V. cholerae*, heat-labile enterotoxin (LT) from *E. coli* and pertussis toxin (PT) from *Bordetella pertussis*. CTX and PT modify G-proteins, also known as guanine nucleotide-binding proteins. G-proteins are heterotrimeric molecules composed by alpha (α), beta (β) and gamma (γ) subunits, and are required to transmit the information from G-protein-coupled receptors (GPCRs) on the plasma membrane to the inside of cells. Depending on the targeted cell types, signals transduced by GPCRs modulate diverse physiological functions, such as the gating of polarisation of cardiac cells, the smooth muscle tone, the carbohydrate and lipid metabolism, immune cell effector functions [189]. CTX and LT bind GM1 ganglioside receptor on the cell surface and then are internalised through the endocytic pathway. In the cytoplasm, the toxin specifically modifies the Gαs at arginine residue 201 [190,191]. The ADP-ribosylated Gαs subunit becomes locked in a GTP-bound state resulting in a constitutive activation of host adenylate cyclase, which produces cyclic-AMP (cAMP). Consequent elevated intracellular levels of cAMP induce activation of protein kinase A (PKA), which in turn phosphorylates and activates the cystic fibrosis transmembrane conductance regulator (CFTR) protein, causing an efflux of ions (sodium and potassium) and water from infected enterocytes into the intestinal lumen, thus leading to severe diarrhea [192]. Similarly to CTX mechanism of action, LT deregulates the ion transport efflux on the luminal surfaces of intestinal epithelial cells, thus favouring intestinal water secretion into the lumen of the bowel and secretory diarrhea [193,194]. Conversely, PT ADP-ribosylates the inhibitory Giα at cysteine 351 residue, which locks the Giα in GDP-bound inactive state, thus leading to a constitutive active cAMP signalling [195–197]. Similar cytotoxic effects have been also described for additional PT-like toxins recently characterised from several *Salmonella species* such as *S. typhimurium (ArtAB-DT104), S. worthington* (ArtAB-SW) and *S. bongori* (ArtAB-Sb) [139,198].

A divergent CTX-like is represented by NarE toxin, which is produced by *Neisseria meningitidis*. This gram-negative bacterium is responsible for meningitis and fulminant septicaemia in humans [199]. Even though NarE toxin shares structural homologies with LT and CTX, it shows different cellular targets. Notably, NarE is a dual enzyme with both ART and NAD^+-glycohydrolase activities. The ART activity of NarE is induced by the presence of target acceptor proteins, such as different cytoskeleton-related proteins, including actin [200]. By contrast, NarE displays a NAD^+ glycohydrolase activity in the absence of substrates [201,202]. At the cellular level, NarE intoxication causes the disruption of epithelial integrity of upper respiratory tract and the induction of apoptotic pathway leading to cell death [200].

bART belonging to C2-like and C3-like toxins converge their activities in modulating, directly or indirectly, the homeostasis of intracellular trafficking processes within the host. The intracellular vesicular transport allows proteins and enzymes to be sorted at specific subcellular compartments through the formation of vesicular intermediates. By doing so, membrane traffic contributes to maintain cell homeostasis [203]. The formation, targeting and fusion of vesicles that transport cargos among different compartments rely on the activity of small GTPases, such as members of the Ras superfamily of monomeric G proteins (Rab) [204,205], the ADP-ribosylating factors (ARFs) [206] and members of the Rho family of GTPases [207]. Intracellular vesicles are transported along cytoskeletal components such as actin filaments or microtubules, which serve a structural role in cell shape and architecture [208,209]. Thereby, MARylation of cellular components of the intracellular transport by bARTs impairs host intracellular trafficking processes, thus sustaining the infection process [210]. The C2-like group includes many toxins from different bacteria among which C2 toxin from *Clostridium botulinum*, Iota toxin from *C. perfringens*, C. difficile toxin (CDT) from *C. difficile*, C. spiroforme toxin (CST) from *Clostridium spiroforme*; VIP2 toxin from *B. cereus*, SpvB from *Salmonella sp*., as well as VgrG1 and AexT from *Aeromonas hydrophila* and *Aeromonas salmonicida*, respectively [211–216]. C2-like exotoxins usually modify actin at Arg177. ADPr of actin leads to the disassembly of actin

filaments and, in turn, impairs cellular motility, migration as well as deregulation of intracellular trafficking [210,217]. For instance, CDT-induced ADPr of actin results in erroneous delivery of intracellular vesicles from the basolateral to the apical side of epithelial cells where extracellular matrix proteins, such as fibronectin, are released, thus promoting host-pathogen interaction [218].

Actin is also a target of TccC3 subunit, a component of the tripartite Tc toxins from *Photorhabdus luminescens*, an emerging gram-negative human pathogen. TccC3 modifies actin at threonine 148, which, contrary to C2-like toxins, induces actin polymerisation and clustering [137].

Members of the C3-like group encompass exoenzymes from *C. botulinum*, *Staphylococcus aureus*, *B. cereus*, *C. limosum* and *Paenibacillus larvae*. C3-like exoenzymes selectively modify the small GTP-binding proteins RhoA/B/C at a conserved Asn residue 41 [140,219–221]. RhoA/B/C GTPases are small-molecular weight G-proteins, which modulate the state of actin cytoskeleton polymerisation. Pathogen-mediated ADPr renders Rho biologically inactive and leads to a lethal redistribution of actin in the target cell [222–224]. Cellular consequences are the alteration of epithelial and endothelial barrier functions [225,226], the impairment of the immune cell signalling including phagocytosis [227]. Rho GTPases are also targeted by TccC5 subunit of Tc toxin from *P. luminescens* [137], though onto different residues (Gln61 or 63). Exoenzyme S and T (ExoS and ExoT) from *Pseudomonas aeruginosa*, which are additional C3-like toxins, are characterised by the presence of a Rho GTPase activating protein (RhoGAP) domain in their sequence [228,229]. ExoS modifies several Rab GTPases, *e.g.* Rab5, Rab7, Rab8, Rab11, thus impairing normal endocytic pathways. For instance, ADPr of Rab5 onto multiple arginine residues (Arg81, 91, 110, and 120) prevents tethering and fusion of endocytic vesicles with early endosomes [230]. As a consequence, plasma membrane-associated receptors, such as epithelial growth factor receptor (EGFR), are trapped in a clathrin-coated vesicle [56]. ExoS also modifies ezrin, radixin, and moesin (ERM) proteins, which are regulators of the actin network through the activation of Rho protein [231]. ADPr of ERM proteins on arginine residues of moesin (Arg553, Arg560 and Arg563) impairs the phosphorylation of ERM protein components operated by the protein kinase C (PKC) and Rho kinases, thus leading to actin cytoskeleton and trafficking alterations. Modulation of ERM functions by ADPr ultimately leads to actin cytoskeleton and trafficking alterations [232,233]. Furthermore, ExoS ADP-ribosylates Ras GTPase at arginine residues (Arg41 and 123) resulting in its inactivation and thus inhibition of Ras signal transduction, which is responsible for cell growth and survival [234].

ExoT recognises two cytosolic proteins as substrates, Crk-I and Crk-II respectively. Both proteins are Src homology 2–3 domains containing adaptor proteins involved in focal adhesion and phagocytosis. ExoT-mediated ADPr of Crk I/II impairs the interaction between Crks and focal adhesion proteins, causing alteration of integrin signalling pathway, which ultimately leads to actin cytoskeleton rearrangement [235,236].

Further, bARTC effectors have been described to modify cellular kinases. The *E. coli* effector EspJ, as well as the SeoC and SboC homologues from *Salmonella enterica* subsp. *salamae* and *arizonae* and *S. bongori*, modifies the highly conserved glutamic residue at position 310 (Glu310) on Src protein to inhibit phagocytosis. In addition, EspJ modifies by ADPr other non-receptor tyrosine kinases, such as the Abl, Csk, Tec, and Syk, leading to inhibition of both autophosphorylation and phosphorylation protein substrates [141,142].

4.1.2. Diphtheria toxin-like

DTX is secreted by *C. diphtheriae* and targets the eukaryotic elongation factor-2 (EF2) as substrate. DTX-dependent ADPr occurs at specific histidine residue (His715); the modified histidine is termed diphthamide. Diphthamide is located in the region of EF2 responsible for the interaction with components of translation machinery, thus ADPr of EF2 acts as an inhibitor of RNA translation and protein synthesis [46,53].

DTX infection leads to necrosis of the heart and liver. The exotoxin A (ExoA) secreted by *P. aeruginosa* as well as the Cholix toxin (ChT) secreted by *V. cholerae* share structural and functional similarities with DTX and both of them modify EF2 with similar functional consequences [237–239].

4.1.3. ADP-ribosylation-dependent ubiquitination as mechanism of pathogenesis

Legionella pneumophila is an opportunistic pathogen that typically spreads via inhaled aerosols and is the causative agent of the so-called Legionnaires' disease, inducing pneumonia. *L. pneumophila* manipulates host functions to achieve the formation of the Legionella-containing vacuole (LCV), a replicative niche necessary for bacterial growth and survival [240–242]. One common strategy adopted by pathogens is the inactivation of ubiquitin-dependent inflammatory signalling pathways [243]. Despite *Legionella* lacks ubiquitination system, it contains a multitude of proteins able to interfere with host ubiquitination machinery. The SidE protein family, which encloses SidE, SdeA, SdeB, and SdeC proteins, contributes to *Legionella* pathogenesis [244,245]. All SidE proteins contain an ARTC-like motif required for their toxicity to yeast [246]. When expressed in mammalian cells, SidE family effectors were found to induce an E1 and E2-independent ubiquitination of several ER-associated Rabs, including Rab33b, Rab1, Rab6a and Rab30 [244]. By clarifying the virulence mechanism of SdeA, it has been shown that the ubiquitination induced by *Legionella* in the host is dependent on ADPr [245]. Structural and biochemical characterisation have revealed that SdeA is a dual enzyme with both MARylation and phosphodiesterase (PDE) activities, due to the presence of two distinct protein folds, namely ARTC-like and PDE domains [245,247,248]. ADPr-dependent ubiquitination of protein substrates catalysed by SdeA is a two-step reaction: first, SdeA transfers ADP-ribose from NAD^+ onto arginine 42 (Arg42) of an Ubiquitin (Ub) molecule in order to generate an ADP-ribosylated-Ub intermediate; in the second step, phosphodiesterase activity converts ADP-ribosylated-Ub intermediate to phosphoribosyl-Ub which is then conjugated through an ester linkage to a serine residue to target protein or to SdeA itself [245,248]. Thus, phosphodiesterase activity of PDE is strictly required to generate a phosphoribosyl-Ub intermediate. The enzymatic step of hydrolysis of the phosphodiester bond within ADP-ribosylated Ub by SdeA resembles the activity displayed by some enzymes belonging to the nucleoside diphosphate-linked moiety X (Nudix) and nucleotide pyrophosphatase/phosphodiesterases (NPP). Enzymes performing such hydrolytic reaction enclose NUDT16 and the bacterial EcRppH as well as ENPP1 and the reptile Phoshodiesterase 1 [249–251].

As a result of SdeA-mediated phosphoribosyl ubiquitination of protein targets, *i.e.* Rab33b, Rab1, Rab6a, Rab30 [244], and ER-associated protein Reticulon-4 (RTN4), the physiological system of ubiquitination cascade is impaired, thus affecting physiological mechanisms requiring this modification, such as mitophagy (Fig. 3) [245,252]. SdeA catalytic mechanism is inhibited by adenosine 5′-O-thiomonophosphate, an AMP analogue, that binds with low-affinity the SdeA PDE domain and alters substrate positioning. This finding provides new insights for a rationale designing of novel antimicrobial compounds to inhibit this class of *Legionella* toxins [248].

SidJ encoded by *L. pneumophila* counteracts SidE toxicity [246,253]. As prolonged activation of SdeA might be also unfavourable for bacterial growth inducing host cell death [254], *Legionella* may have evolved SidJ as a deubiquitinase enzyme described for its ability to hydrolyse the phosphodiester linkage of phosphoribosyl-ubiquitinated substrates [255].

4.2. Endogenous bacterial ADPr and cell homeostasis regulation

Cell homeostasis is ensured by the activity of metabolic enzymes,

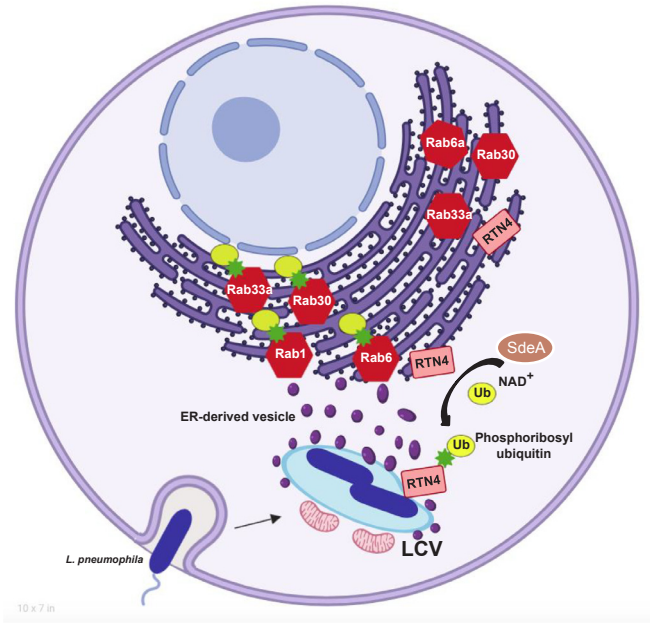

Fig. 3. Schematic representation of *Legionella pneumophila* infection mediated by SdeA effector. By transferring a phosphoribosyl group on ER-resident substrates, such Reticulon-4 (RTN4) and several Rab proteins, SdeA induces the formation of ER-derived vesicles and their association with the Legionella-containing vacuoles (LCVs). LCVs also make contacts with ER and mitochondria, a phenomenon that affects physiological processes, such as ER-phagy and mitophagy.

regulated according to environmental conditions. As such, nitrogenase reductase enzymatic activity, responsible for nitrogen fixation (*i.e.* the conversion of nitrogen to ammonium), is modulated through endogenous MARylation in the photosynthetic nitrogen-fixing bacteria *Rhodospirillum rubrum* and *Azospirillum brasilense* [168,256,257]. This mechanism has been extensively studied in the alphaproteobacteria and bioinformatics analysis supports the idea that a similar mechanism is active in at least 25 bacterial genera [257,258]. The reaction is catalysed by the dinitrogenase reductase ADP-ribosyl transferase (DraT). DraT shows a very low sequence similarity with bacterial ARTD and ARTC members, suggesting the idea that new enzymes may contribute to expand the role of ADPr.

DraT modifies the dinitrogenase reductase at arginine 101, which leads to enzymatic activity loss and shutting-off the nitrogen fixation. The reversal of DraT-dependent ADPr is performed by a specific ADP-ribosylarginine glycohydrolase (DraG), thus fully restoring dinitrogenase reductase activity [168]. The DraT and DraG activities are subjected to opposite regulation *in vivo*. In response to negative stimuli, such as exposure to darkness or high concentrations of ammonium, DraT promotes the inactivation of nitrogenase by ADPr. Conversely, when the negative stimulus is removed, namely light illumination or exhaustion of ammonium in turn, the enzymatic activity of DraG is stimulated, thus restoring nitrogenase function [259].

Mechanisms of ADPr are also part of toxin-antitoxin (TA) systems, potent tools exploited by bacteria in order to modulate fundamental bacterial processes in response to environmental stimuli. TA systems are indeed required to induce dormancy and persistence in bacteria under hostile environmental conditions and to form biofilms as well [260–262]. TA systems are also involved in the pathogenesis of several infectious diseases caused by *M. tubercolosis* [263], *E. coli* [264], *Haemophilus influenzae* [265], and *S. typhimurium* [266]. Toxin-antitoxin (TA) modules consist of a stable toxin and of a cognate anti-toxin, mostly encoded in an operon, whose co-expression is co-regulated at transcriptional and translational level. An ADPr-dependent TA system is encoded by the pathogen *M. tuberculosis*. The toxin DarT MARylates the second thymidine base within the TNTC conserved sequence of single-stranded chromosomal bacterial DNA. As a result of this modification, DarT interferes with DNA replication or transcription, consequently leading to bacterial growth arrest and dormancy. DNA modification is reversed by the cognate anti-toxin macrodomain-enzyme (DarG). As DarTG system induces bacteriostatic effects, it represents a promising candidate in drug design for developing novel anti-virulence strategies [8].

Additional TA systems involving ADPr encompasses ParST encoded by the bacterium *Sphingobium* sp. YBL2 [267]. ParT exerts bacteriostatic effects *via* ADPr of phosphoribosyl pyrophosphate synthetase (Prs), an essential enzyme in nucleotide biosynthesis conserved in all organisms. The bacteriostatic state is reversed by interaction with the cognate anti-toxin ParS, which does not act as a hydrolase enzyme.

Further, Tre1 (type VI secretion ADP-ribosyl transferase effector 1) from *Serratia proteamaculans* is a novel bARTC toxin with a role in inter-bacterial species competition; it has been recently reported to modify the critical microbial cell division protein FtsZ, the tubulin-like protein. Following the modification, FtsZ loses its ability to polymerise resulting in the alteration of cytoskeletal structure and leading to cell death. The ARH-like hydrolase Tri1 can revert the modification [268].

5. The BFA-mediated ADP-ribosylation-like reaction

Brefeldin A (BFA) has been described as a non-canonical ADPr toxin [269]. Although BFA is a non-bacterial toxin, we discuss it because of its peculiar mechanism of ADPr. BFA was indeed isolated as macrocyclic lactone from fungal organisms such as *Eupenicillium brefeldianum* and *Alternaria carthami*, where it is synthesised starting from palmitate [270]. BFA has been shown to induce ADPr of two eukaryotic proteins; GAPDH and a protein of 50 kDa [269], which was named BARS (BFA-ADP-ribosylation substrate). BARS was later identified as a member of C-terminal-binding protein-1 short-form (CtBP1-S/BARS), member of the CtBP family [271]. CtBP1-S/BARS is a protein involved in two specific functions, one in the cytoplasm and the other in the nucleus [272]. In the cytoplasm, CtBP1-S/BARS is involved in membrane fission that controls the formation of post-Golgi carriers [273–275] endocytic fluid-phase carriers [276], and COP1-coated vesicles [277], and it is also involved in mitotic Golgi partitioning [278]. In the nucleus, members of the CtBP protein family act as transcription co-repressors, and thus regulate numerous cellular functions, including epithelial differentiation, tumourigenesis and apoptosis [279]. The BFA-dependent ADPr of CtBP1-S/BARS is a non-classical two-step reaction [280]. The first step is a novel form of ADPr of a small molecule resulting from the covalent binding of ADP-ribose derived from NAD^+ or cyclic ADP-ribose to BFA. This reaction leads to the formation of a BFA-ADP-ribose conjugate (BAC). ADP-ribosyl cyclases, such as CD38, are the enzymes responsible for the synthesis of BAC, which happens in the extracellular space. In a second step, BAC efficiently internalises through the CD38 channel-like dimeric structure and conjugates with NAD(H)-binding Rossmann fold of CtBP1-S/BARS [280,281]. According to the model, the C3 atom of BFA falls in close proximity with the imidazole ring of His304 of CtBP1-S/BARS [280]. Notably, the proposed mechanism for BAC binding to CtBP1-S/BARS, which relies on the His304/Glu289/Arg86 (H-E-R) triad, is in agreement with the structural similarities between CtBP1-S/BARS and D2-hydroxy acid dehydrogenases [281], where the structurally equivalent H-E/D-R triad is involved in substrate binding and dehydrogenase activity. Of note, BAC selectively binds the Rossmann fold of BARS, but not that of other dehydrogenases [280]. From the functional point of view, the new BFA-dependent ADPr reaction affects the cytoplasmic fission-inducing activity of CtBP1-S/BARS. BAC impairs the binding of CtBP1-S/BARS to partners involved in fission activity, thereby inhibiting BARS-dependent mitotic Golgi fragmentation. This event results in a potent and prolonged cell-cycle block in G2 phase of the cell cycle [280].

Notably, in addition to the cytotoxic effects mediated by ADPr, BFA

also acts as a non-competitive inhibitor of the ARF guanine nucleotide exchange factors (GEFs). BFA binds the transient ternary complex that is formed by ARF, GDP and the GEF Sec7 [282] inducing the release of a set of proteins from the Golgi complex [283]. Based on the latter property, BFA has been used as a research tool to analyse the mechanisms of intracellular membrane transport. Indeed, the main effects of BFA are a dramatic morphological reorganisation of the Golgi complex and redistribution of both resident and cargo proteins from the Golgi membranes to the ER [284–286]. BFA also affects the morphology and function of the endosomal/lysosomal compartments [287]. BFA has shown several biological and potent activities such as antiviral [288], antifungal [289], antitumoural and apoptosis-inducing properties in several cancer cell lines [290–292].

6. Conclusions: targeting toxin ADP-ribosyl transferase activity

Antibiotics have been the treatment of choice for bacterial diseases for long time, however, because of the growing bacterial resistance to antibiotics, alternative solutions have been searched to help limit the severity of the infection [64]. Novel antimicrobial approaches rely on targeting specific bacterial toxins causative of virulence instead of impairing bacterial processes common with non-pathogenic resident microbiota. In the last decade, strategies raised against toxins have been developed according to their diverse mechanism of action, for instance by inhibiting enzymatic activities of toxins [65,293]. Targeting of toxin ART activity mainly relies on chemical modulation of NAD^+-interaction pocket inside the ART domain to block enzymatic activity. Initial approaches have foreseen the use of PJ34, a non-specific inhibitor, largely known for targeting human PARPs. Although PJ34 has been shown to inhibit efficiently *P. aeruginosa* exotoxin-A (K_i = 140 nM), it was also able to target endogenous PARPs in the mouse models of stroke, therefore lacking the specificity to ART toxins, needed for therapeutic administration. In order to address the specificity of inhibitors for bART, drug design approach studies have been performed by using the co-crystal structure of DXT or ChT in complex with PJ34 as template for virtual screening [294]. 500,000 commercially available molecules and a small library of twelve known PARP inhibitors have been analysed. Such approach led to the identification of a set of lead compounds capable of inhibiting ChT and ExoA, and CTX exotoxin ART activity both *in vitro* and in cell-based assays [294]. Further, additional strategies have instead exploited the antimicrobial protection provided by polyphenolic compounds from grape extract [295]; the screening of individual compounds has led to identify twelve molecules active against CTX, where four out of twelve acting through inhibition of ART activity. Similarly, molecules inhibiting ExoS ART activity with an IC_{50} of 1.3 μM have been proposed as starting point for a precise targeting of virulence factors [296]. The high conservation of ADPr mechanisms throughout the evolution suggests that in-depth studies are needed in order to ensure that therapeutic molecules targeting bacterial toxins would not be specific for endogenous mechanisms of ADPr in the host, whose alteration may cause serious side effects.

Exploitation of bacterial Toxin-Antitoxin systems relying on ADPr mechanism could represent an innovative strategy to fulfil the requirement of new, non-canonical targets. TA systems have no human counterparts, are widespread in bacterial genomes, thus representing alternative targets for development of antimicrobial compounds. Of note, inhibition of the anti-toxin component (such as DarG), either by the administration of selective inhibitors or by silencing (*i.e.* by sequence-specific antisense agents), could be envisaged as a novel therapeutic strategy [297].

However, despite the great efforts in developing anti-virulence compounds or in applying available PARP inhibitors for targeting ART activities involved in infectious diseases, the use of blocking antibodies still represents the gold standard treatment for neutralising bacterial toxins [63,64,298–300].

Declaration of Competing Interest

The authors declare no conflict or financial interests.

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